MOLECULAR MECHANISMS OF *ARABIDOPSIS* RESISTANCE TO GREEN PEACH APHID, *MYZUS PERSICAE*

A Dissertation

by

JIAXIN LEI

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Keyan Zhu-Salzman
Co-Chair of Committee,	Libo Shan
Committee Members,	Hisashi Koiwa
	Xiuren Zhang
Interdisciplinary Faculty Chair,	Dirk B. Hays

December 2014

Major Subject: Molecular and Environmental Plant Sciences

ABSTRACT

Aphid, a specialized phloem sap-feeding insect, is one of the major pests of a number of economic important crops, including soybean, cotton, and maize. Aphid can have devastating effects on the crop production by limiting plant growth, as well as serving as vectors for disease. Therefore, research on identification of plant resistance mechanisms to aphid is important for crop improvement. Here, I have developed a system consisting of *Arabidopsis thaliana* and green peach aphids (*Myzus persicae*) to study plant-aphid interaction. In my research project, the role of BIK1 was investigated in *Arabidopsis* infested with the green peach aphid. Loss of BIK1 function adversely impacted aphid settling, feeding and reproduction. Relative to wild-type plants, *bik1* displayed higher aphid-induced H_2O_2 accumulation and more severe lesions, resembling a hypersensitive response (HR) against pathogens. Basal as well as induced salicylic acid and ethylene accumulation were in in the *bik1* mutant. Intriguingly, elevated salicylic acid levels did not contribute to the HR-like symptoms or to the heightened aphid resistance associated with the *bik1* mutant. Elevated ethylene levels in *bik1* accounted for an initial, short-term repellence. Introducing a loss-of-function mutation in the aphid resistance and senescence-promoting gene *PHYTOALEXIN DEFICIENT4* (*PAD4*) into the *bik1* background blocked both aphid resistance and HR-like symptoms, indicating *bik1*-mediated resistance to aphids is PAD4-dependent. Taken together, *Arabidopsis* BIK1 confers susceptibility to aphid infestation through its suppression of

PAD4 expression. Furthermore, the results underscore the role of ROS and cell death in plant defense against phloem sap-feeding insects.

ACKNOWLEDGEMENTS

Special thanks to my advisor, Dr. Keyan Zhu-Salzman for her guidance and supervision over the course of my research. Special thanks also go to my committee members, Dr. Hisashi Koiwa, Dr. Libo Shan, and Dr. Xiuren Zhang for their guidance and support. I also want to extend my gratitude to Dr. Scott Finlayson, Dr. Bhimu Patil, Dr. G. K. Jayaprakasha, Dr. Rammohan Uckoo, Dr. Michael Kolomiets and Dr. Eli Borrego for the GS-MS and LC-MS analyses. I also would like to thank my formal and current labmates, fellow MEPS students, and the department faculty and staff for providing their support and making my Ph.D study in Texas A&M University a great experience. Finally, thanks to my family for their encouragement and love.

NOMENCLATURE

ABA	Abscisic Acid
BIK1	*Botrytis*-Induced Kinase1
CCA1	Circadian Clock Associated1
COI1	Coronatine Insensitive1
EE	Evening Element
EIN2	Ethylene Insensitive2
EIN3	Ethylene Insensitive3
ET	Ethylene
GLS	Glucosinolate
HR	Hypersensitive Response
JA	Jasmonic Acid
LHY	Late Elongated Hypocotyl
LUX	LUX Arrhythmo
PAD4	Phytoalexin Deficient4
PRR	Pseudo-Response Regulator
ROS	Reactive Oxygen Species
SA	Salicylic Acid
TOC1	Timing of Cab Expression1
SID2	Salicylic Acid Induction Deficient2
ZT	Zeitgeber Time

ZTL	Zeitlupe
I3M	Indol-3-ylmethyl
4MO-I3M	4-Methoxyindol-3-ylmethylglucosinolate
1MO-I3M	1-Methoxyindol-3-ylmethyl

TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

Page

LIST OF TABLES

1. INTRODUCTION AND LITERATURE REVIEW*

1.1. Green peach aphid, *Myzus persicae,* is an important agriculture pest

Myzus persicae, also known as green peach aphid, is one of the most agricultural important aphid species across the North America and worldwide. Green peach aphid is exceptional in many aspects. First, *Green peach aphid* is extremely polyphagous and feeds on a wide range of host plants in over 40 families, including several families of important vegetable crops, such as Chenopodiaceae, Compositae, Cruciferae, Cucurbitaceae, and Solanaceae. Second, the major economic cost of green peach aphid is due to its high efficiency as a virus vector. It can transmit numerous plant viruses, including both persistent and non-persistent viruses. For instance, persistent viruses beet western yellows virus, beet mild yellowing virus, as well as non-persistent viruses cucumber mosaic virus and bean yellow mosaic virus are transmitted by green peach aphid (Hogenhout et al., 2008). Third, the physiological and ecological traits of green peach aphid strains, such as body color, life cycle, and relationships with host plants, display a great range of genetically-based variability (Van Emden and Harrington, 2007). Populations adapted to tobacco (*Nicotiana tabacum*) are quite distinct from other populations (Takada, 1986). They grows fast, mainly colonizes on meristem tissues and youngest leaves, and appealingly somehow avoid or tolerate the glandular trichome

---

* Part of this chapter is reprinted with permission from **Lei J, Finlayson SA, Salzman RA, Shan L and Zhu-Salzman** (2014) *BOTRYTIS*-INDUCED KINASE1 modulates arabidopsis resistance to green peach aphids via PHYTOALEXIN DEFICIENT4. Plant Physiology **165**: 1657-1670. The journal URL (www.plantphysiol.org).

exudates, which contain toxic, as well as repellent compounds (Emden et al., 2007). Forth, green peach aphid shows striking capability to develop resistance to more insecticides than any other known species. Report show that green peach aphid is resistant to more than 200 different insecticides (Georghiou and Lagunes-Tejeda, 1991). These characteristics, combined with the capacity of the rapid growth of insect population, have made this aphid one of the most economic important agricultural pests in the Unite State.

1.2. The interaction between aphid and *Arabidopsis thaliana*

Aphids are specialized to feed and survive on phloem sap of their host plants. In contrast to chewing insects that cause extensive plant tissue damage, aphids have evolved to manipulate resource allocation within the host plant by converting the feeding site into a sink to deplete photoassimilates (Girousse et al., 2005). Their highly modified stylets navigate through plant tissues predominantly intercellularly before reaching phloem, causing very limited host cell damage. During probing and feeding, aphids secrete gelling and watery saliva (Tjallingii, 2006). Gelling saliva forms the sheath enveloping the stylet along the pathway leading to the vascular bundle. The sheath limits damage to plant cells and avoids triggering extracellular defenses. Watery saliva is thought not only to prevent clogging of phloem sieve elements and the food canal in aphid stylets due to protein coagulation, but also to modulate host cellular processes and mitigate host defense (Tjallingii, 2006; Will and van Bel, 2006; Will et al., 2007). Aphids make use of their stealthy feeding strategies and intimate associations with their

hosts to disguise themselves and overcome plant defense, reminiscent of the deceptive strategies frequently employed by pathogens (Kaloshian, 2004; Walling, 2008).

1.2.1. *Aphids reprogram host plant metabolism*

The damage of aphid on plant growth is mainly due to depletion of photosynthetic products by feeding on phloem sap in the sieve element. Aphids reprogram host plant metabolisms by alternation of source-sink relationship, promoting flow of nutrients to the infested tissues (Girousse et al., 2005; Louis and Shah, 2013). Aphid not only affects expression of genes related carbohydrate biosynthesis, metabolism, and transportation (Moran and Thompson, 2001; Moran et al., 2002), but also results in accumulation of sucrose, trehalose and starch (Singh et al., 2011; Hodge et al., 2013). Research suggests that the accumulation of starch at the insect feeding sites, at the expense of sucrose, is critical for plant defense against aphid by creating "a secondary intracellular sink" to cope with aphid-imposed physiological changes. *Arabidopsis phosphoglucomutase1* (*pgm1*) mutant, deficient in starch biosynthesis, is more susceptible to aphid (Singh et al., 2011). In rice (*Oryza sativa*), higher accumulation of starch is observed in the line resistant to brown planthopper (*Nilaparvata lugens*), a phloem sap-feeding insect; while, a susceptible line contains less starch (Hao et al., 2008). Moreover, trehalose biosynthesis-related mutant *trehalose synthase11* (*tps11*) supports more aphid than wild-type plant. Since trehalose promotes production of starch in plants, this result further supports the role of starch in plant resistance to aphids (Singh et al., 2011).

Nitrogen (N), involved in the biosynthesis of amino acids and nucleic acid, is essential for growth and development for most organisms. Since aphids feed on diets generally poor in N nutrition, N contents are critical for their survival. Aphid infestation dramatically impacts host plant nitrogen metabolism and allocation. Aphid feeding increases amino acid levels in infested leaves (Dorschner et al., 1987). In alfalfa (*Medicago sativa*) stem, pea aphid (*Acyrthosiphon pisum*) infestation triggers re-allocation of N from apex to stem, converting a sink tissue into a source tissue to obtain more nutrients (Girousse et al., 2005). Aphid also alters N stable-isotope signature of host plant by inducing nitrate reductase activity (Wilson et al., 2011). In spite of altered amounts of amino acid in host plants affect aphid feeding activity, no significant effect on aphid reproduction is detected (Hunt et al., 2006; Hunt et al., 2010).

1.2.2. *Host plant defense against aphids*

During the long history of co-evolution, plants have developed sophisticated means to protect themselves against assaults from various herbivorous insects. Most plants are equipped with constitutive and induced defense mechanisms including physical barriers, such as trichomes and cell walls, and chemical defense, such as secondary metabolites. Despite the deceptive feeding style of aphids, the brief intracellular punctures along the stylet passage and secretions from salivation nevertheless trigger responses in host plants (Tjallingii, 2006; Will and van Bel, 2006; De Vos and Jander, 2009; Bos et al., 2010).

Plant defense responses can be classified as antibiosis, which curtails insect survival and reproduction, and/or antixenosis, which deters insect settling and herbivory. Transcriptomic studies suggest that phloem sap feeders modulate known defense signaling pathways, oxidative stress response, senescence, and plant metabolism and structure (Moran and Thompson, 2001; Zhu-Salzman et al., 2004; De Vos et al., 2005; Thompson and Goggin, 2006; Kusnierczyk et al., 2008).

Plant response to aphids involves genes regulated by the major plant hormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA), and genes encoding transcriptional regulators. Exogenous JA application enhances plant resistance to aphids (Ellis et al., 2002; Zhu-Salzman et al., 2004; Cooper and Goggin, 2005). Furthermore, reduced population expansion was observed in Green peach aphid when raised on the *Arabidopsis constitutive expression of vegetative storage protein 1* (*cev1*) mutant constantly expressing JA responses, whereas the JA-insensitive mutant *coronatine-insensitive1* (*coi1*) supports more rapid growth of aphids than WT plants (Ellis et al., 2002; Mewis et al., 2005). Aphid infestation has been shown to trigger ET production (Mantelin et al., 2009). Elevated ET levels have been both positively and negatively correlated with plant resistance to aphids (Thompson and Goggin, 2006). In tomato, ET biosynthesis renders plants more susceptible to potato aphids (*Macrosiphum euphorbiae*) (Mantelin et al., 2009). However, the *Arabidopsis* ethylene-insensitive mutant *ein2* promotes performance of green peach aphids (Kettles et al., 2013), indicating that ET plays a defensive role in *Arabidopsis*. Aphid feeding activates the SA signaling pathway in a number of plant species (Moran and Thompson, 2001; Moran et

al., 2002; Zhu-Salzman et al., 2004). SA-mediated resistance to aphids has been observed on some occasions (Mohase and van der Westhuizen, 2002; Kaloshian, 2004), but SA does not seem to play a defensive role in *Arabidopsis* against aphids (Pegadaraju et al., 2005). ABA has also been implicated as a modulator of plant immunity via signaling crosstalk (Fujita et al., 2006; Koornneef and Pieterse, 2008). Mutations in ABA biosynthesis and signaling have significant impacts on aphid population growth (Kerchev et al., 2013). Comparison of plant gene expression profiles reveals that aphid feeding and pathogen infection induce both similarly and differentially regulated gene sets (Barah et al., 2013).

The localized cell death elicited by microbial pathogens known as the hypersensitive response (HR) is considered a defense mechanism used by plants to prevent further spread of infection (Torres et al., 2006). A hallmark of hypersensitivity in many plants is local production of reactive oxygen species (ROS), such as H_2O_2. HR-like symptoms, manifested as localized chlorotic and necrotic lesion spots, can also be detected in plants attacked by various insect herbivores. Strong HR-like symptoms, including rapid and prolonged accumulation of H_2O_2, were detected in lines of wheat (*Triticum aestivum*) resistant to Hessian fly (*Mayetiola destructor*), but not in the susceptible line (Liu et al., 2010). Enhanced resistance against phloem sap-sucking brown planthopper (*Nilaparvata lugens*) is accompanied by increased H_2O_2 levels as well as HR-like cell death in rice (*Oryza sativa*) expressing an antisense lipoxygenase (Zhou et al., 2009). Oxidative stress induced by insect herbivory is considered a component of soybean (*Glycine max*) resistance to invading corn earworm (*Helicoverpa*

zea) (Bi and Felton, 1995). *Arabidopsis* PHYTOALEXIN DEFICIENT4 (PAD4), a lipase-like protein essential for defense against microbial pathogens (Jirage et al., 1999), has been demonstrated to enhance plant resistance to green peach aphid (*Myzus persicae*) by promoting premature leaf senescence and cell death (Pegadaraju et al., 2005; Pegadaraju et al., 2007). Functional dissection further revealed that the molecular mechanism of PAD4 resistance against aphids is distinct from that against pathogens (Louis et al., 2012).

Glucosinolates are a family of secondary metabolites mainly occurred in the *Brassicaceae* family, including *Arabidopsis*, as well as several important vegetables, such as broccoli, cabbage, and oilseed rape. The basic structure of glucosinolates contains three core parts: an S-linked β-glucopyranosyl residue, an O-linked sulfate residue, and a side chain derived from vary amino acids (Griffiths et al., 2001). Glucosinolates are categorized into three major groups based on the amino acid precursor of the side chain. Aliphatic glucosinolates are derived from methionine, aromatic glucosinolates are derived from phenylalanine or tyrosine, and indolic glucosinolates are derived from tryptophan (Sonderby et al., 2010). Glucosinolates promote host plant resistance to insect herbivores (Hopkins et al., 2009). The defensive properties of glucosinolates mostly come from their breakdown products. The hydrolyzing myrosinase is a thioglucosidase stored in specific cells (Rask et al., 2000). When plants are damaged by a chewing insect, both glucosinolates and myrosinases are released from the cells and act together to produce some toxic compounds, such as isothiocyanates, nitriles, and oxazolidinethiones (Bones and Rossiter, 2006). JA,

wounding and chewing insect treatments induce production of glucosinolates (Brader et al., 2001; Mewis et al., 2005; Mewis et al., 2006). However, the mode of action of glucosinolates on plant defense against aphids is different from chewing insects. As mentioned earlier, aphids avoid activation of myrosinase by minimizing wounding to plant cells. Thus plants cannot produce the active forms of glucosinolates. Moreover, aphid infestation suppresses both expression of glucosinolate biosynthesis-related genes and accumulation of most glucosinolates, with the exception of one indolic glucosinolate, 4-methoxyindol-3-ylmethylglucosinolate (4MO-I3M) (Kim and Jander, 2007). Although aphids do not trigger hydrolysis of indolic glucosinolates in plants, their breakdown products are detected inside the insects, suggesting that aphids can metabolize indolic glucosinolates in their bodies. Interestingly, the aliphatic glucosinolates are intact in the insects (Kim et al., 2008). Indolic glucosinolates have antifeedant effects on aphids, shown in both feeding on artificial diets containing individual indolic glucosinolates, and tests using *Arabidopsis* mutant line with elevated production of indolic glucosinolates. These studies indicate that post-ingestive breakdown of indole glucosinolates can be an effective defensive mechanism against aphids, which avoid myrosinases-dependent activation of glucosinolates in plants.

1.2.3. *Metabolic resistance of aphid adaptation to host plant defensive compounds*

While plants employ a broad spectrum of defense mechanisms (Howe and Jander, 2008), insects show extraordinary phenotypic plasticity by utilizing behavioral, physiological and biochemical strategies to cope with host plant resistance (Brattsten,

1988). Metabolic resistance, often resulted from overproduction of detoxification enzymes, is the primary mechanism insect utilizes to degrade toxins (Brattsten, 1988). Catalases (CATs), antioxidant enzymes, directly convert H_2O_2 into water (DeJong et al., 2007). Glutathione S-transferases (GSTs) detoxify secondary oxidation products which are generated during ROS reaction with intracellular macromolecules, such as DNA, proteins and lipids (Hayes and Pulford, 1995; Hayes and McLellan, 1999). GST is one of the key enzymes for aphid adaptation to plant defensive secondary metabolites, including glucosinolate (Francis et al., 2005). Carboxylesterases (COEs) catalyze hydrolysis of various substrates with a carboxylic ester. Despite few studies have proved the direct role of COEs in ROS detoxification, they involve in metabolisms of many toxins, indicating their putative role in ROS product elimination (Despres et al., 2007).

1.3. Circadian clock

1.3.1. *Critical concepts in circadian clock*

In nature, most organisms profoundly change their metabolism, physiology, and behavior from day to night in response to the daily shift between day and night. Circadian rhythms are the endogenously generated and self-sustaining biological rhythms with periods of 24 h. Circadian clock enables organisms to anticipate different times of the day and tune their body to the changes of external environments without exogenous cures, such as light/day cycles. Therefore, circadian rhythms are normally studied under constant environments, including constant light and temperature. Critical

concepts commonly used in studies of circadian rhythms are as follows (Fig. 1-1). Period is determined by the time to complete one cycle from peak to peak. Zeitgeber means 'time giver' in German. Zeitgeber time (ZT) is the time of day during a rhythm. Phase is defined as the time of day for the peak. For instance, if a rhythm peak at 3 h after sunrise, the phase would be defined as ZT3. Amplitude measures the magnitude of changes between peak and trough during a period (McClung, 2006).

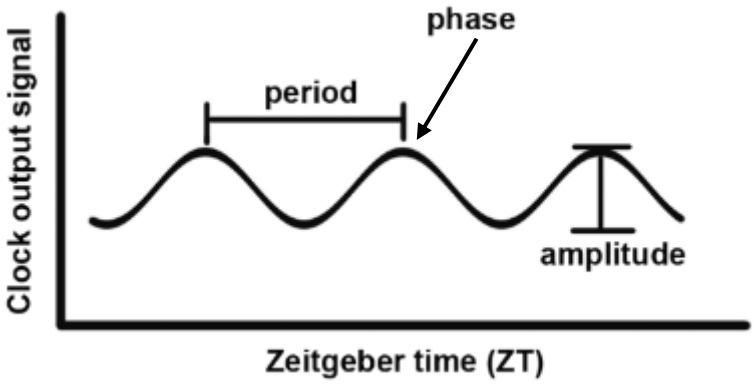

Figure 1-1. Critical parameters used in studies of circadian rhythms.
Period, phase, amplitude, as well as zeitgeber time (ZT), are presented. (Adapted and modified from Figure 1 in Mclung, et al. 2006)

The circadian system model consists three major components: input pathways, oscillator, and output pathways. Input pathways are defined as "the sequence of events via which information from the environment, such as changes in light and temperature, is transduced to the oscillator" (Barak et al., 2000). Oscillators are the core components of the clocks, which can be defined as "the cell-autonomous timekeeper responsible for generating self-sustained rhythmicity" (Barak et al., 2000). Output pathways are "the

processes linking the oscillator with the various biological activities it controls" (Barak et al., 2000), including oscillation of clock-regulated gene expression and leaf movement rhythms.

1.3.2. *Core components in circadian clock*

In *A. thaliana*, circadian clock is an intricate signaling network which consists of interlocked feedback loops among molecular components (Fig. 1-2). Circadian clock contains central, morning, and evening loops. In the center loop, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), two morning-expressed Myb transcription factors (Schaffer et al., 1998; Wang and Tobin, 1998; Mizoguchi et al., 2002), both positively and negatively regulate genes containing the Evening Element (EE) motif (Harmer and Kay, 2005). CCA1 and LHY repress the expression of an evening-expressed transcription factor, TIMING OF CAB EXPRESSION1 (TOC1), by directly binding to the EE motif in the *TOC1* promoter (Alabadi et al., 2001). Reciprocally, TOC1 also inhibits the expression of *CCA1* and *LHY* (Gendron et al., 2012; Huang et al., 2012). TOC1 belongs to a group of family called the PSEUDO-RESPONSE REGULATORS (PRRs), with PRR5, PRR7 and PRR9, which repress the expression of *CCA1* and *LHY* in the morning loop (Huang et al., 2012). The morphological phenotypes and metabolic profiles of the triple mutant of *PRR5*, *PRR7* and *PRR9*, *prr579*, are similar to the *CCA1* overexpressing line (*CCA1-ox*) (Fukushima et al., 2009). ZEITLUPE (ZTL), as an F-box protein functions in evening loop, negatively regulates TOC1 expression by targeting its degradation (Kim et al.,

2003; Mas et al., 2003). The expression of *CCA1* and *LHY* are down-regulated in *ztl* mutant (Baudry et al., 2010). LUX ARRHYTHMO (LUX), an evening-expressed Myb transcription factor, indirectly activates *CCA1* expression by repressing expression of *PRR9* (Helfer et al., 2011). Lower expression of *CCA1* and *LHY* are detected in *lux* (Hazen et al., 2005).

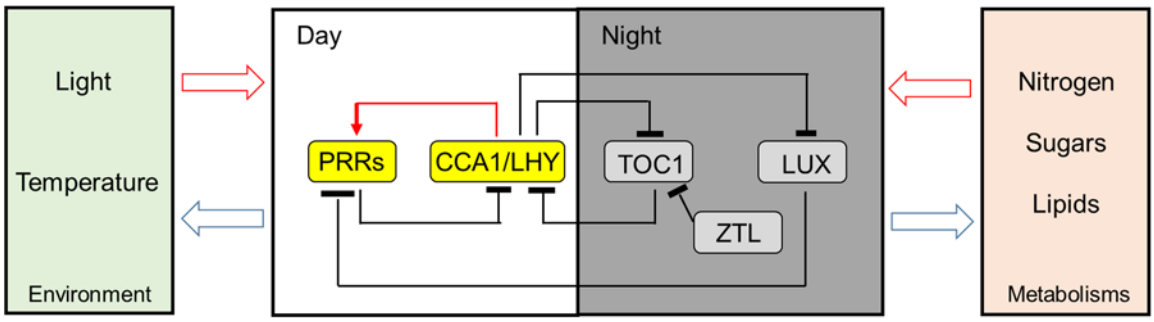

Figure 1-2. A simplified working model of the circadian clock in *Arabidopsis*. CCA1 and LHY act as repressors for *TOC1* expression. Reciprocally, TOC1 also down-regulates the expression of CCA1 and LHY. *PRR*s are activated by CCA1 and LHY in the morning; in return, the expression of *CCA1* and *LHY* are repressed by PRRs. ZTL, an F-box protein, degrades TOC1 protein with E3 ubiquitin ligase SCF complexes. LUX, which is also repressed by CCA1 and LHY, negatively regulates PRRs. Red arrows lines indicate activation, while black lines indicate repression. Plant circadian clock system also actively interacts with external environment, as well as its metabolic profiles.

1.3.3. *The interplay between circadian clock and plant primary and secondary metabolisms*

Plant primary and secondary metabolisms, which are required for normal growth, development and reproduction, dynamically respond to external environments, including light, temperature, water, as well as abiotic and biotic stresses. Recent transcriptomic

and metabolomic studies demonstrate a wide spread circadian regulation of various metabolic processes. In return, these metabolic processes often affect the clock, as well as its output.

Recent studies indicate that the biosynthesis of nitrogen and carbohydrate are regulated by circadian clock. The central clock component CCA1 alters mRNA abundance of *glutamine synthetase* (*GLN1.3*) and *glutamate dehydrogenase* (*GDH1*) by targeting their promoter regions (Gutierrez et al., 2008). In *Arabidopsis*, most amino acids reach peak levels during dusk under light/dark (LD) cycles, but few of them is rhythmic under constant light (Espinoza, et al. 2010). Similar to those in LD cycles, the amino acids which oscillate in constant light also peak at subjective dusk (Espinoza et al., 2010). Inorganic and organic N (e.g. glutamine and glutamate) in turn act as input signal to affect circadian clock (Gutierrez et al., 2008). Moreover, *Arabidopsis* displays oscillations in carbohydrate levels which also provide metabolic feedback signaling to circadian clock (Haydon et al., 2013). Circadian clock co-regulates transcript levels of photosynthesis genes which peak at midday (Harmer et al., 2000; Blasing et al., 2005). Accordingly, carbohydrates (e.g. glucose and sucrose), as the principal photosynthetic products in plants, also peak in the middle of the day (Harmer et al., 2000; Haydon et al., 2013).

Circadian clock regulates pathways involved in phytohormone biosynthesis and signaling. In tobacco, the abundance of cytokinin (CK), indole-3-acetic acid (IAA) and abscisic acid (ABA) show diurnal variation (Novakova et al., 2005). Ethylene (ET) emission in *Arabidopsis* is under circadian control and peaks at dawn in constant light

(Thain et al., 2004). While JA and SA often work antagonistically, they oscillate with circadian rhythms and peak at opposite phases (Goodspeed et al., 2012). In agreement with hormone levels, global transcriptome analysis indicates that genes response to hormones, including JA, SA, ABA, ET and IAA, are circadian-regulated and peak at specific time of the day (Covington et al., 2008). In return, key parameters of circadian clock are also influenced by phytohormones, including CK, IAA and ABA, but not SA and ET (Hanano et al., 2006).

Glucosinolates, a group of sulphur-containing secondary metabolic compounds involved in plant resistance to microbe pathogen and insect herbivores, are regulated by circadian clock. In both cabbage and *Arabidopsis*, total glucosinolate amounts start to increase at dawn and peak at midday, and then begin to rapidly decline at dusk. Individual members of glucosinolates behave differentially. Several indolic glucosinolates, such as I3M, 4MO-I3M, and 1MO-13M, accumulate to higher levels in day than night with varying amplitude and magnitude (Goodspeed et al., 2013). Recent genomic and metabolomic quantitative trait loci analyses identify naturally variable loci that links altered circadian clock outputs with natural variation of glucosinolate amounts among different *Arabidopsis* accessions (Kerwin et al., 2011). Both expression of clock-related genes (e.g. *CCA1*, *LHY*, *PRR7*, and *PRR9*) and circadian periods are significantly altered in mutants of glucosinolate biosynthesis genes.

2. *BOTRYTIS*-INDUCED KINASE1 NEGATIVELY REGULATES *ARABIDOPSIS* RESISTANCE TO GREEN PEACH APHIDS THROUGH SUPPRESSING HYPERSENSITIVE RESPONSE*

2.1. Introduction

Over the past several decades, immense progress has been made in research on the early signaling events during plant perception of microbes. A number of membrane receptors as well as intracellular *Resistance* (R) genes, which facilitate plant perception of specific microbe-derived and plant-derived molecules, are reported and studied for their molecular and biochemical functions (Jones and Dangl, 2006). Basal disease resistance, the first line of plant defense response, is elicited upon detection of pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) by specific transmembrane pattern-recognition receptors and is collectively termed PAMP-triggered immunity (Boller and Felix, 2009; Monaghan and Zipfel, 2012). Among the best characterized *Arabidopsis* PAMP/MAMP receptors are receptor-like kinases (RLKs) such as FLAGELLIN-SENSITIVE2 (FLS2) that recognizes bacterial flagellin, and EF-TU RECEPTOR (EFR) that recognizes bacterial elongation factor EF-Tu (Gomez-Gomez and Boller, 2000; Zipfel et al., 2006). Upon binding to their cognate MAMPs, FLS2 or EFR associate with another RLK, BRI1-ASSOCIATED RECEPTOR KINASE

(BAK1) (Chinchilla et al., 2007). *BOTRYTIS*-INDUCED KINASE1 (BIK1), a receptor-like cytoplasmic kinase (RLCK), is directly phosphorylated by BAK1 and associates with FLS2/BAK1 complex in modulating PAMP-mediated signaling (Lu et al., 2010; Zhang et al., 2010; Liu et al., 2013). Most recently, BAK1 is shown to be required for aphid elicitor-mediated ROS induction and plant innate immunity to aphids (Prince et al., 2014). Likewise, TOMATO PROTEIN KINASE1b (TPK1b), the tomato homolog of BIK1, plays an important role in plant resistance to a chewing insect herbivore (Abuqamar et al., 2008). The second layer of plant defense response is mediated by plant disease resistance (R) proteins, which recognize specific avirulence proteins from pathogens. R gene-mediated resistance to aphids has been reported although the corresponding avirulence proteins from aphids remain unknown (Kaloshian, 2004). The tomato R gene *Mi-1* confers resistance to some biotypes of potato aphids (*Macrosiphum euphorbiae*), as well as to whiteflies (*Bemisia tabaci*) and root-knot nematodes (*Meloidogyne incognita*) (Rossi et al., 1998; Vos et al., 1998; Nombela et al., 2003).

Rapid and accurate responses to insect herbivory in host plant is essential to successfully implement induced defenses. During the plant-insect interaction, the process of herbivore recognition relies on early signaling events, which occurs well before changes of defense-related transcriptomic and metabolic profile in host plant (Howe and Jander, 2008). Compared to studies on pathogen recognition, less studies have conducted plant recognition of aphid infestation. Plant defense response to aphid infestation and bacterial pathogen infection share some common signatures. As in plant defense against pathogens, gene-for-gene interaction for aphid resistance is evident.

Recent studies also implicate that aphid triggered signaling events could potentially interface with PAMP-mediated signaling. Bos et al. (2010) selected 48 candidate effector genes from the salivary gland of green peach aphid based on common features of plant pathogen effectors. Transient overexpression in plants suggested that some putative effectors could suppress oxidative burst induced by flg22 and changed plant defense response. Thus it is tempting to speculate that some components involved in initial recognition and signaling in response to aphids and pathogens might be similar.

In this study, we examined the roles of several RL(C)Ks, including FLS2, EFR, BAK1, and BIK1, in *Arabidopsis* response to aphid infestation. We challenged these loss-of-function mutants with Green peach aphid to evaluate aphid performance and plant response. *bik1* plants displayed heightened antibiosis and antixenosis toward aphids, which was correlated with pronounced aphid-induced HR-like cell death. Further exploration of potential interactions between BIK1 and known defense pathways revealed that BIK1 modulated plant response to aphid infestation through its control of PAD4 expression.

2.2. Materials and methods

2.2.1. *Plant growth and aphid rearing*

Arabidopsis thaliana was grown in LP5 potting medium (Sun Gro Horticulture, Bellevue, WA) in environmental chambers at 23ºC (day) /21ºC (night), 65% relative humidity (RH) and 12L/12D photoperiod with a photosynthetic photon flux density of 85 μMoles $m^{-2}s^{-1}$. For plant damage evaluation, histochemical assays and aphid no-

choice tests, 4 to 5-week-old plants were used. For plant gene expression analyses and hormone measurements, as well as for aphid choice tests, 3 to 4-week-old plants were used.

Phloem sap-feeding green peach aphids *M. persicae* (a tobacco-adapted red lineage, kind gift from Dr. Georg Jander, Boyce Thompson Institute for Plant Research, Cornell University, NY) were cultured on cabbage (*Brassica oleracea*) and maintained in an environmental chamber at 21ºC, 65% RH, and 12L/12D photoperiod (63 μMoles $m^{-2}s^{-1}$). All insect treatments and bioassays were performed in this chamber.

2.2.2. *Arabidopsis lines*

The previously reported *Arabidopsis* lines, wild-type Col-0 and mutants *fls2 (SALK_141277)*, *fls2 (SALK_062054)*, *efr*, *bak1-3*, *bak1-4*, *bik1*, *sid2*, *nahG, bik1 sid2, bik1 nahG*, *ein2-1*, *ein3-1*, *pad4*, *bik1 pad4* and the *bik1* complementation line *bik1+BIK1* used in this study (Fig. 2-1) (Jirage et al., 1999; Veronese et al., 2006; Lu et al., 2010; Laluk et al., 2011; Lin et al., 2013) were kindly provided by Dr. T. Mengiste at Purdue University or obtained from the Arabidopsis Biological Resource Center, Ohio State University. To generate *bik1 ein2-1* and *bik1 ein3-1* double mutants, we crossed *bik1* with *ein2-1* and *ein3-1* respectively using *bik1* as the female parental line. The F2 seeds were germinated in the dark on Murashige and Skoog agar medium containing 50 μM 1-aminocyclopropane-1-carboxylic acid. The seedlings that lacked a triple response were selected and transferred to soil. The presence of *ein2-1* and *ein3-1* was confirmed by the derived cleaved amplified polymorphic sequence (dCAPS) method as previous

described, with modification (Nandi et al., 2003; Binder et al., 2007; Bouchez et al., 2007; Chen et al., 2009). For *ein2-1* genotyping, a 195 bp fragment flanking the point mutation was amplified by PCR, followed by purification and A*fl*II restriction digestion. A*fl*II cut the mutant sequence into 160 bp and 35 bp fragments but left the WT sequence intact. For *ein3-1*, the 222 bp PCR product remained intact in the mutant sequence but was cut by *Hae*III into 190 bp and 32 bp fragments in the WT sequence. DNA fragments were resolved on 2% agarose gel. For *bik1* genotyping, a procedure developed previously was followed (Lu et al., 2010). The sequences of primers used for genotyping were shown in Table 2-1.

Table 2-1. Primers used in this study.

Gene name	Accession number	Sense primer (5'→3')	Antisense primer (5'→3')
Quantitative RT-PCR			
ERF1	AT3G23240	CAAGACCTTCCGATCAAATCCGT	CCCGAGCCAAACCCTAATACC
PDF1.2	AT5G44420	TGTTTGGCTCCTTCAAGGTT	TTCTCTTTGCTGCTTTCGAC
PR1	AT2G14610	CGTTCACATAATTCCCACGAG	TCAGTGAGACTCGGATGTGC
MYC2	AT1G32640	TGAAGAAGATAAAGCAAACCCGA	TCCTGTACTCCTGATCCGCC
PAD4	AT3G52430	TCTTCAGTTAAAGATCAAGGAAGG	GGTTGAATGGCCGGTTATC
SAG13	AT2G29350	GCCCACCCATTGTTAAAAGC	ACGACTCCAGCAGCAGAGGAT
UBQ10	AT4G05320	AGATCCAGGACAAGGAAGGTATTC	CGCAGGACCAAGTGAAGAGTAG
Genotyping			
EIN2	AT5G03280	GTTTGAGATGGAATACCGTGATGG	TCAAGGATCGCAGATAAGTGTCTCC
EIN3	AT3G20770	TACCAAGTATCAAGCGGAG	AGGCCACCAATCCTCTTTC

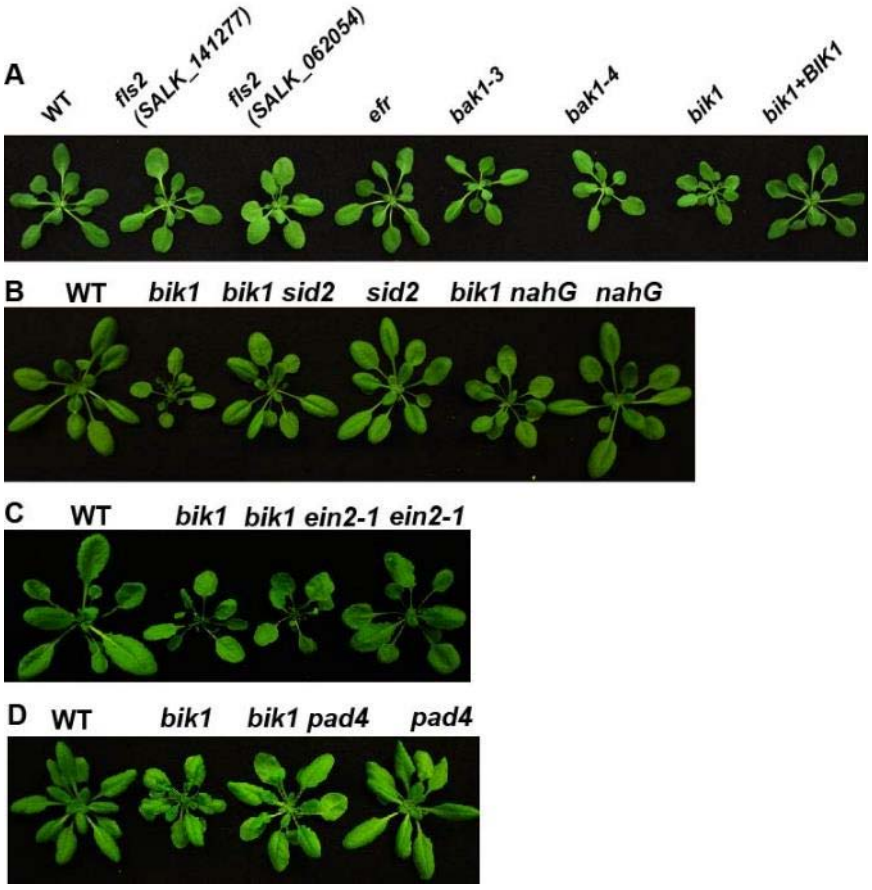

Figure 2-1. Mature shoot phenotypes of various *Arabidopsis* genotypes used in the study.
Shoot phenotypes of **(A)** 4 to 5-week-old WT, *fls2 (SALK_141277)*, *fls2 (SALK_062054)*, *efr*, *bak1-3*, *bak1-4*, *bik1* and *bik1+BIK1 Arabidopsis*, and of mutants used in plant damage assays in evaluation of effects of **(B)** SA, **(C)** ET, and **(D)** PAD4 on *bik1*-mediated hypersensitivity and resistance to aphids.

2.2.3. *Insect bioassays*

Aphid no-choice and choice tests were performed to assess the antibiotic and antixenotic resistance of different *Arabidopsis* genotypes. For the no-choice tests, 6 age-synchronized second instar nymphs (within 24 h) were placed on 4-week-old plants.

The total aphid population (adult and nymph) on each plant was counted 7 days after infestation. Each genotype had at least 10 replicates. For the choice tests, 35 adults were released at an equal distance between two plants of different genotypes. The number of adult aphids settled on each plant was recorded 6 and 24 hours after releasing. At least 10 pairs of plants were used in each comparison. All experiments were repeated at least three times and a representative data set was presented.

To obtain the average adult aphid body weight, adult aphids were transferred to WT or *bik1* plants and removed 24 h later to produce age-synchronized progenies. Ten days later, the new generations of adults reared on *Arabidopsis* genotypes were collected and were weighed as 6 groups of 10 aphids each.

Eggs of fall armyworm, purchased from Benzon Research Inc (Carlisle, PA), were incubated in a growth chamber (27°C and 65% RH). Newly hatched larvae were transferred to 4-week-old WT or *bik1* plants. Plants were replaced once a week to ensure sufficient food supply. Larvae reared on *Arabidopsis* genotypes were weighed after feeding for 16 or 22 days. At least 30 larvae were measured for each genotype.

2.2.4. *Ninhydrin staining and quantification of aphid honeydew*

Honeydew production served as an indicator of insect feeding activity. To determine honeydew secretion, Whatman filter papers, protected by a plastic membrane to avoid absorbance of water from soil, were placed under *Arabidopsis* plants of various genotypes infested by 30 adult aphids. These filter papers were collected 1, 2 and 3 days after aphid infestation, soaked in 0.1% ninhydrin in acetone, and dried in a 65ºC oven for

30 min. Honeydew stained by ninhydrin was shown as purple spots (Kim and Jander, 2007).

To quantify the honeydew stains, the filter papers were cut into pieces and stains were extracted into 1 mL of 90% methanol for 1 h at 4ºC with continuous agitation. After centrifugation at 6,000 g for 1 min, the absorbance of the supernatant was measured at 500 nm (Nisbet et al., 1994). Methanol (90%) served as a blank.

2.2.5. *Plant damage and histochemical assays*

Four to five-week-old *Arabidopsis* plants were infested with adult aphids taking into consideration the variation of the rosette size of each genotype. Accordingly, 48 aphids were placed on WT, *fls2*, *efr*, *bak1-3*, *bak1-4*, *bik1+BIK1*, *sid2*, *nahG*, *ein2-1*, *ein3-1* and *pad4* (sizes comparable to WT), 12 on *bik1*, *bik1 ein2-1* and *bik1 ein3-1* (one quarter the size of WT), and 24 on *bik1 sid2*, *bik1 nahG* and *bik1 pad4* (one half size of WT). Plants were examined daily to identify symptoms of yellowing and lesion formation. Digital images were taken of representative leaves at 6-days post aphid infestation. Leaves obtained in the same manner were subjected to histochemical assay (see below). For every experiment, eight plants or more of each genotype were used. All experiments were repeated at least 3 times.

To visualize H_2O_2 accumulation, 3,3'-diaminobenzidine (DAB) staining was performed. Leaves at 6-days post infestation, as well as control leaves, were collected and vacuum-infiltrated with DAB solution (1 mg/ml DAB, in pH 3.5 water) in a 6-well titer plate. After an overnight incubation in the same solution in darkness, the leaves

were destained in 95% ethanol until they turned clear. Images were then captured with a digital camera.

To determine local and systemic ROS accumulation, aphids were placed in clear plastic cups (4 cm diameter, 4 cm height) with mesh cloth replacing the bottoms for ventilation. Twenty insects were used for WT, and 10 for *bik1*. The cage was fitted around the leaf petiole between the cap and the cup, and sealed with cotton to avoid wounding as well as aphid escape, restricting the aphids onto one 4-week-old *Arabidopsis* leaf for the desired time (Kim and Jander, 2007). Caged leaves without aphids served as controls. After treatments, the cages were removed and leaves were excised for DAB staining.

Trypan blue staining was performed to visualize cell death. Trypan blue was dissolved in lactophenol solution (phenol: lactic acid: glycerol: water [1: 1: 1: 1]) at a concentration of 0.125 mg/mL. Leaves prepared as above were boiled in this staining solution for 1 min. After cooling, leaf samples were destained in 95% ethanol, and photographed with an Olympus SZX2-ILLK microscope (Olympus Corporation, Tokyo, Japan).

The accumulation of autofluorescent compounds and deposition of callose are features of HR lesions (Hunt et al., 1997). Lesions on *Arabidopsis* leaves were examined 6 days after aphid infestation using the Olympus microscope under bright field or UV excitation with a green fluorescent protein (GFP) filter. Images of lesions and autofluorescence emitted from the same lesion sites were recorded (Stewart et al., 2009).

Aniline blue staining (Clay et al., 2009) was performed to detect callose deposition. *Arabidopsis* leaves were fixed in buffer containing 10% formaldehyde, 5% acetic acid, and 50% ethanol at 37°C overnight. Slightly translucent leaves were then washed in 95% ethanol several times until clear, rinsed twice in water, and then stained for 4 hr or longer in the dark with 0.01% aniline blue in 150 mM K_2HPO_4 (pH 9.5). Callose deposits were visualized with an Olympus IX-81 microscope at 10x magnification under UV illumination with a broadband DAPI filter set.

2.2.6. *JA, SA and ABA measurements*

For SA, JA and ABA measurements, 3-week-old plants were infested with aphids (30 per plant). Two days later, treated or control plants were ground to a fine powder in liquid nitrogen. For each sample replicate, ground tissue (60 mg) and a mixture of stable isotope-labeled hormones including 10 ng $^2H_4$-SA, 3.8 ng $^{13}C_2$-JA, and 1 ng of $^2H_6$-ABA were added to a 5 mL glass tube with 500 µL of methanol at 55ºC, and extracted by vortexing three times during a 10 min incubation. The tissue was re-extracted with 500 µL methanol, and then once with 500 µL of 80% ethanol warmed to 55ºC, centrifuging and pooling the cleared supernatants after each extraction. The pooled extracts were dried and the residue was resuspended in 800 µL of chloroform and partitioned against 1 mL of H_2O adjusted to pH 9.0 with NH_4OH. The aqueous fraction was recovered, adjusted to pH 5.0 with acetic acid and partitioned against 1 mL of ethyl acetate. The organic fraction was transferred to a Reactivial, dried, and then methylated with ethereal diazomethane. Samples were then analyzed on an Agilent

7890A/7693A/5975C XL GC-MS equipped with a 0.25 mm x 30 M DB-5MS column (0.25-μm film) using pulsed splitless injection. Helium was used as the carrier gas at 0.75 mL/min. The inlet was maintained at 250°C and the oven was ramped from 45°C (2.25 min initial hold) to 250°C at 40°C per minute, held at 250°C for 3 min, and then ramped to 290°C at 40°C per min. The ion source temperature was maintained at 230°C and the quadrupole was heated to 150°C. The ion source was operated in electron impact mode and both scan and selected ion data were acquired. Two ions were monitored for each hormone, and the larger fragment was used for peak area quantification (SA- 120,124,152,156; JA- 193,195,224,226; ABA- 162,166,190,194 m/z).

2.2.7. *ET measurement and 1-MCP treatment*

Three-week old *Arabidopsis* were infested with aphids (30 per plant) for 2 days. Shoots were excised, weighed and kept in 10 mL-syringes with 3-way stopcocks to seal them. One hour later, 1 mL of headspace gas was injected into a Photovac 10SPlus gas chromatograph (Photovac, Markham, Ontario, Canada). At least 6 individual plants were averaged for each treatment. Each experiment was repeated at least three times. ET was quantified by integration of peak area, relative to an authentic standard (Finlayson et al., 2007).

1-methylcyclopropene (1-MCP) gas was generated by dissolving a solid formulation of a proprietary 1-MCP α-cyclodextrin complex (AgroFresh) in 0.1N NaOH in a flask fitted with a septum. The mass of the 1-MCP α-cyclodextrin complex used

was calculated to produce 1000 ppm 1-MCP gas in the headspace of the flask. An aliquot of the concentrated 1-MCP gas was then injected into a desiccator to give a final calculated concentration of 1 ppm. Plants in the desiccator thus were subjected to 1-MCP treatment. After 1 h exposure to 1-MCP, plants were brought to a normal environmental atmosphere. This procedure was repeated every 12 h for 5 days to maintain the effect of 1-MCP, followed by aphid choice tests. Control plants were handled in the same manner without 1-MCP gas.

2.2.8. *Quantitative RT-PCR*

Plant samples were harvested, frozen and ground in liquid nitrogen to a fine powder. Total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) and then treated with RNase-free DNase (QIAGEN, Valencia, CA). Equal amounts of RNA (2 μg) were used to synthesize cDNA with random hexamer primers and SuperScript™ II Reverse Transcriptase (Invitrogen). Quantitative RT-PCR (qRT-PCR) reactions were performed using SYBR Green Mastermix (BioRad, Hercules, CA) according to the manufacturer's protocol. Primers were designed using PerlPrimer software (Marshall OJ, 2004), and their quality was examined using NCBI Primer Blast. Primer sequences are provided in Table 2-1. *Arabidopsis UBQ10* (AT4G05320) served as an internal control for data normalization. qRT-PCR was run on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Controls using untranscribed RNA confirmed that there was no genomic DNA contamination. Dissociation curve

analyses were applied to check amplification specificity. The mean fold change in gene expression was calculated as described previously (Zhu-Salzman et al., 2003).

2.2.9. *Statistical analysis*

SPSS 16.0 software (SPSS Inc, Chicago IL) was used for analyses of all data. The no-choice tests of aphid performance among genotypes were analyzed by one-way ANOVA. Tukey's multiple range test analysis was used for pairwise comparisons of the difference between treatments for mean separation ($P < 0.05$). The Chi-square test was applied to the aphid choice tests ($P < 0.05$).

2.3. Results

2.3.1. *bik1 plants exhibited enhanced resistance to green peach aphids*

Plant defense response upon aphid infestation is often reflected by reduced offspring production (antibiosis) in a no-choice test with reduced feeding and body weight, or by non-preference (antixenosis) in a choice test. To determine whether the several known RL(C)Ks, which play important roles in PAMP-triggered immunity, extend their function to aphid-associated defense response, we evaluated aphid performance on the loss-of-function mutants (Fig. 2-2; 2-3). Aphids infesting *fls2, efr* and *bak1* mutants had fecundities comparable to that on the wild-type (WT) Col-0 plants (Fig. 2-2A). Likewise, no particular preference was detected among them (Fig. 2-2C), suggesting that these RLKs may not play a major role in plant defense against aphids. Interestingly, on *bik1*, the amount of aphid progeny was on average about half that on

WT plants (Fig. 2-2A). In agreement with this no-choice test result, aphids on *bik1* excreted less honeydew (Fig. 2-2D), indicative of less food intake, and had less body weight (Fig. 2-2B) than those reared on WT. In the choice tests, approximately twice as many aphids preferred WT versus *bik1* plants (Fig. 2-2C). Thus, BIK1 was a negative regulator of plant resistance to aphids. In addition, we confirmed that the heightened resistance in *bik1* is indeed due to loss of BIK1 function via complementation experiments. Transgenic plants expressing *BIK1* cDNA in *bik1* mutant recovered the susceptibility to aphids in both choice and no-choice tests (Fig. 2-2E), verifying that the observed aphid resistance in *bik1* was due to loss of *BIK1* function.

Notably, *bik1* mutant showed comparable size and biomass during the first 3 weeks of growth (Fig. 2-2C; Table 2-2), when choice tests were performed. Later, *bik1* mutant exhibited growth defect and were smaller than WT (Fig. 2-1; Table 2-2). However, the antibiotic activity was unlikely due to their small stature, as inoculating six 2nd instar nymphs and rearing them for 7 days on 4 to 5-week-old plants would by no means result in a population limited by space or nutrients.

Although *BIK1* is highly induced by pathogens (Veronese et al., 2006), we did not detect a significant change in *BIK1* expression upon aphid infestation (Fig. 2-3F). This is further supported by published microarray data (Couldridge et al., 2007; Kusnierczyk et al., 2007; Kusnierczyk et al., 2008).

Table 2-2. Loss of BIK1 function negatively affects growth and reproduction traits in *Arabidopsis*

	Age	WT	*bik1*	*P*-value
Above-ground biomass[1] (mg)	23 d	2.07 ± 0.10	2.61 ± 0.22	*NS*[2]
	33 d	21.55 ± 1.03	12.63 ± 0.92	0.003 **
	43 d	98.87 ± 7.50	39.54 ± 2.43	< 0.001 ***
Rosette diameter (mm)	23 d	31.61 ± 1.46	32.18 ± 1.53	*NS*
	33 d	73.38 ± 2.07	48.19 ± 2.61	< 0.001 ***
	43 d	96.47 ± 2.32	62.88 ± 2.78	< 0.001 ***
Days to bolting		40.85 ± 1.14	32.6 ± 0.4	< 0.001 ***
Total rosette leaf number[3]		24.85 ± 2.08	14.6 ± 0.3	< 0.001 ***
Total number of filled siliques/plant[4]		228.2 ± 33.4	45.8 ± 10.4	< 0.001 ***
Silique length[5] (mm)		9.15 ± 0.32	4.25 ± 0.31	< 0.001 ***

[1] Average dry weight of all above-ground tissues

[2] NS: not significant

[3] Total number of rosette leaves at the start of bolting

[4, 5] Plants scored for siliques were 12 weeks old

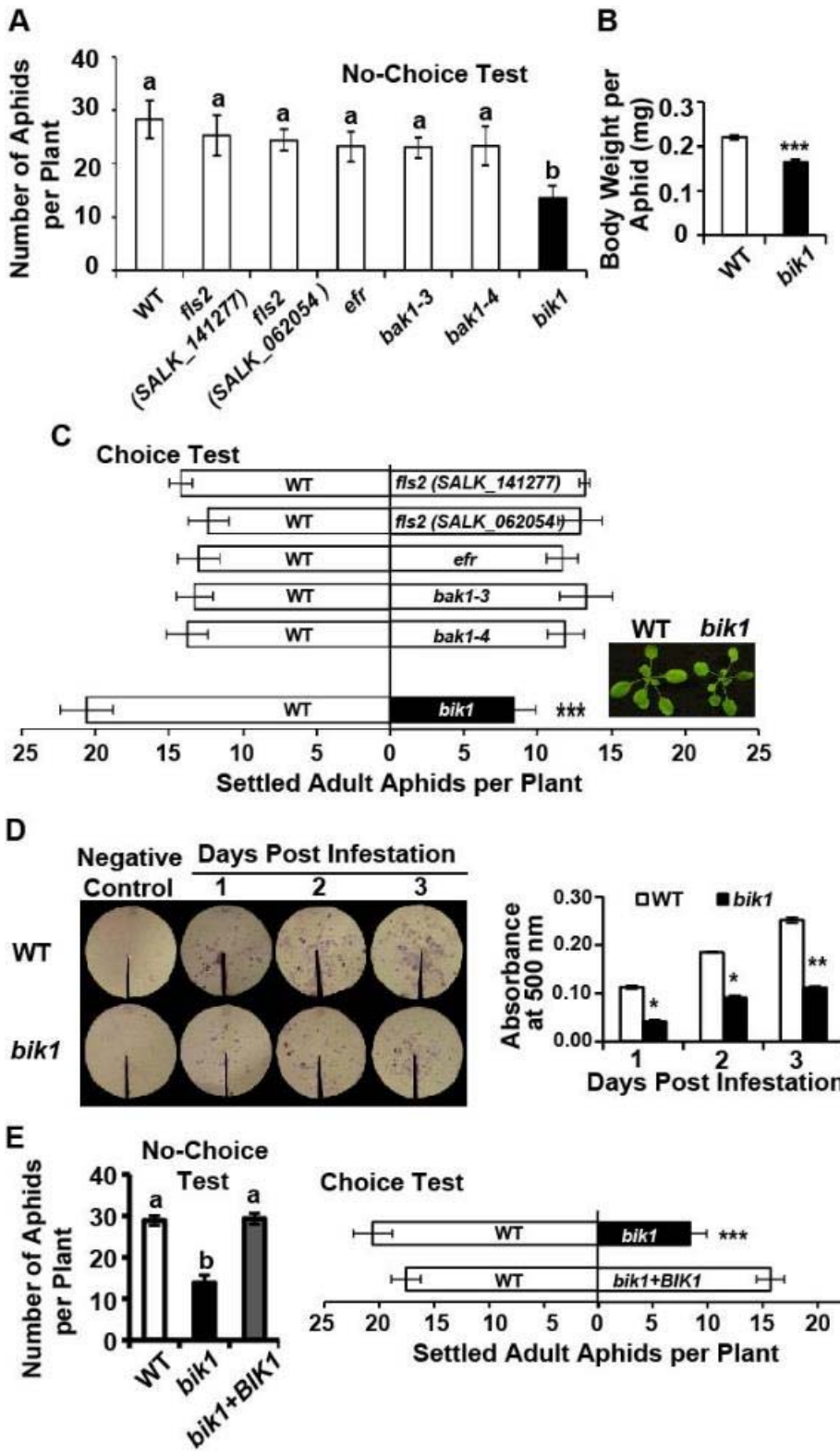

Figure 2-2. Loss of BIK1 function confers resistance to green peach aphids.
(A) No-choice tests and **(B)** aphid body weight of indicated genotypes. For no-choice tests, 6 second instar nymphs were inoculated on each plant (4-5 weeks old). Total aphid numbers were recorded 7 days later. At least 10 replications were performed for each genotype. To obtain average body weight of adult aphids, neonates were reared on WT or *bik1* for 10 days. Adults were then collected and were weighed as 6 groups of 10 aphids each. **(C)** Choice tests. Three-week old plants were used. At this developmental stage, no apparent size differences were observed between genotypes including the WT vs. *bik1* pair. Settled aphids were counted 6 h after releasing 35 adults in between two plants of the tested genotypes. Each test was comprised of 10 replicates. Inset image of the shoot phenotypes of 3-week old, uninfested WT and *bik1*. **(D)** Aphids on *bik1* excreted less honeydew than those reared on WT. Quantity of honeydew secretion was correlated with the area and intensity of ninhydrin stains (left) and with OD_{500} values (right). **(E)** Expression of *BIK1 cDNA* confers WT levels of aphid susceptibility to *bik1*. One-way ANOVA was applied to no-choice tests and the Chi-square test was used to analyze data derived from choice tests. Body weight and honeydew secretion data were analyzed by independent samples t-test. Bars represent means ± standard error (SE). Statistical significance for treatment effects is marked *****($P < 0.05$), ******($P < 0.01$) or *******($P < 0.001$). Means with different letters were significantly different ($P < 0.05$).

2.3.2. *Aphids induced hypersensitive response (HR)-like lesions in bik1*

Despite an enhanced resistance to aphid infection, *bik1* began to show apparent lesion spots approximately 5 days after aphid infestation, while no visible lesions were observed in *fls2*, *efr* and *bak1* mutants or in WT (Fig. 2-3A). With continued aphid infestation, all infested plants, regardless of the genotype, eventually displayed stunted growth, yellowing and necrosis with lesions spreading to the entire leaf and the whole plants. Notably, *bik1* is not a lesion mimic mutant as no spontaneous lesions were observed without aphid infestation. Since *bik1* plants are dwarfs, the number of aphids applied was adjusted by a ratio proportional to the rosette area. For plant symptom assessment, this ratio was applied for all genotypes exhibiting size differences relative to WT, to exclude potential misjudgment due to size discrepancies.

We further examined whether the aphid-induced lesion formation in the *bik1* mutant resembles the features with an HR process that is often correlated with plant resistance against microbial pathogens (Lamb and Dixon, 1997; Heath, 2000). Using 3,3'-diaminobenzidine (DAB) staining, we observed that leaves of aphid-infested *bik1* plants had much higher H_2O_2 accumulation than any other genotypes examined (Fig 2-3B). Likewise, more severe cell death was shown in aphid-infested *bik1* leaves compared with WT and the other mutants by the trypan blue staining assay (Fig. 2-3C). In contrast, *fls2*, *efr* and *bak1* mutants showed phenotypes similar to WT plants in either H_2O_2 or cell death assays. Furthermore, we detected accumulation of autofluorescent phenolic compounds and deposition of callose at necrotic spots in aphid-infested *bik1* plants (Figs. 2-3D, 2-3E), which are also HR lesion-associated histological markers

(Hunt et al., 1997; Luna et al., 2011; Williams et al., 2011). WT levels of H_2O_2 and lesions upon aphid infection were restored in the *bik1 BIK1* complementation line (Fig. 2-3). Taken together, the data indicate that aphid-induced lesions in *bik1* were an HR-like response.

2.3.3. *Heightened local accumulation of ROS and expression of genes involved ROS biosynthesis and signaling in bik1*

Since cellular H_2O_2 accumulation precedes cell death (Hoeberichts and Woltering, 2003), earlier time points were chosen for DAB staining. Staining became apparent within 3 hours upon aphid infestation in *bik1* leaves, but was absent from the infested WT leaves over the 24 hour course of the experiment (Fig. 2-4A). When aphids were caged on specific leaves, H_2O_2 could only be detected in infested local leaves, not in uninfested systemic leaves (Fig. 2-4B), supporting our conclusion that the lesion formation in *bik1* is an HR rather than a constitutive plant damage phenotype. Correlation between plant symptoms and aphid performance suggests that elevated H_2O_2 accumulation and cell death in *bik1* could be the defense mechanism compromising aphid fitness. BIK1 thus functions to counteract aphid-induced ROS production and cell death, distinct from its role in PAMP pathways.

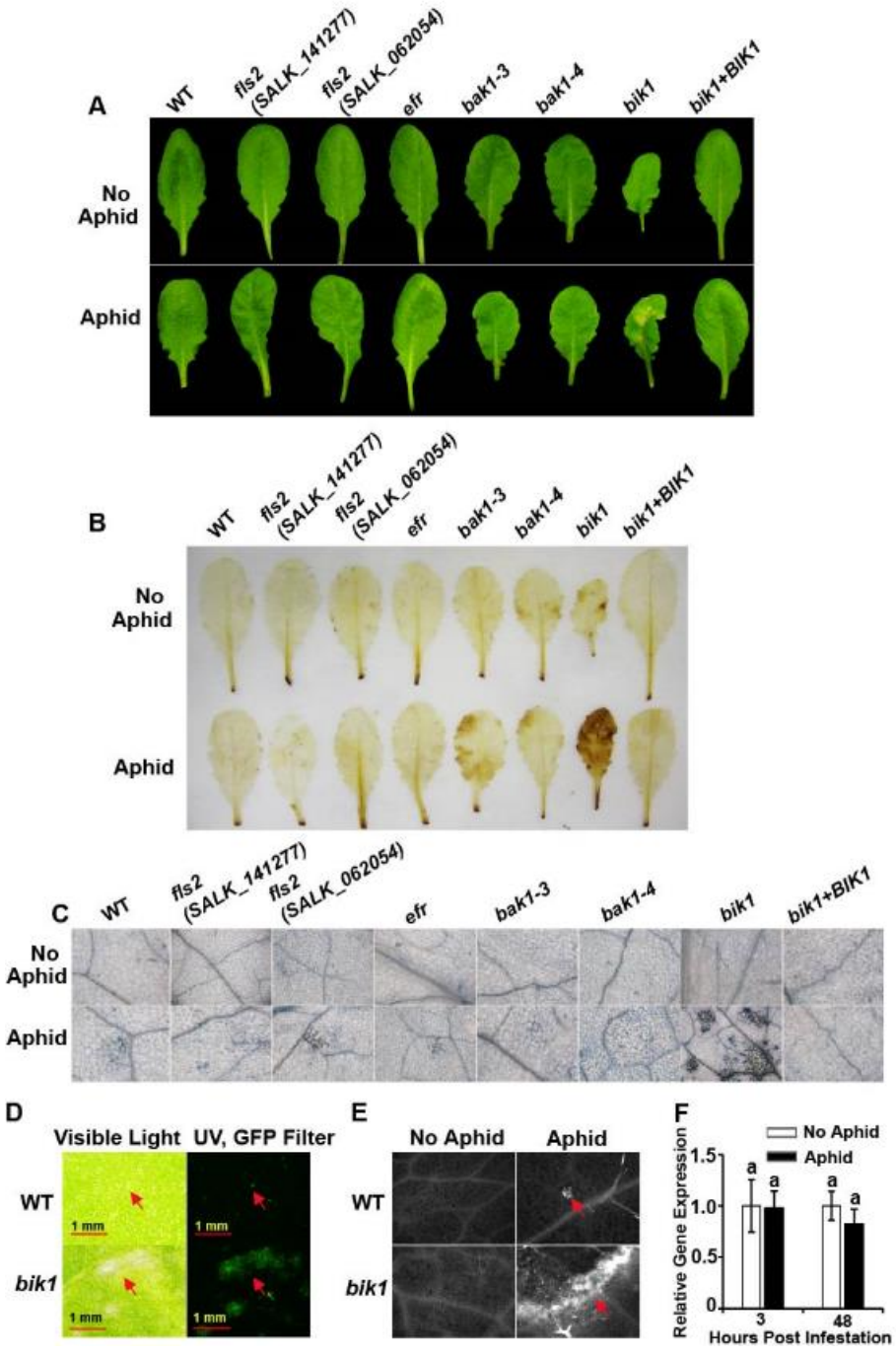

Figure 2-3. Aphid infestation induces a heightened hypersensitive response in *bik1*.
Representative leaf images of **(A)** lesion formation, **(B)** DAB staining (H_2O_2 indicator) and **(C)** trypan blue staining (cell death indicator) prior to (top panel) or 6 days after (bottom panel) aphid infestation of genotypes indicated. **(D)** Autofluorescence of aphid-induced lesion spots under UV excitation with green fluorescent protein filter set (right). The same fields of view are shown under visible light (left). **(E)** Callose deposition at lesion sites. Left: control leaves; right: callose deposition after aphid treatment. Arrows point to lesion sites. **(F)** Relative expression of *BIK1* in WT plants in the presence and absence of aphid infestation. Three-week-old plants were infested with aphids as described in Materials and Methods. Data were analyzed by independent samples t-test. Means with different letters were significantly different ($P < 0.05$).

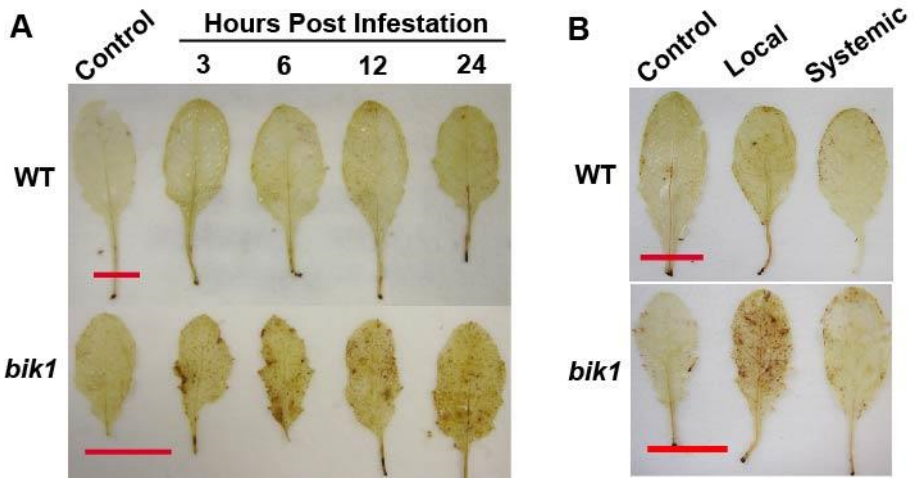

Figure 2-4. *bik1* exhibits earlier and stronger ROS accumulation in locally infested leaves compared with WT.
(A) DAB staining (H_2O_2 indicator) of aphid-infested leaves collected at 3, 6, 12 and 24 h post infestation. Four-week-old *Arabidopsis* plants were infested with aphids using the caged-leaf method as described in Materials and Methods. Caged (24 h) but uninfested leaves served as a control. Scale bars =1.0 cm. **(B)** DAB staining of local, infested and systemic, uninfested leaves of the same plant at 24 h post infestation. All leaves were caged. Controls were caged leaves from uninfested plants. Experiments were repeated 3 times.

Rapid H_2O_2 production upon aphid infestation in bik1 mutant suggests that BIK1 affects ROS homeostasis. *Arabidopsis RESPIRATORY BURST OXIDASE HOMOLOGUE* (*AtRBOH*) genes are NADPH oxidases involved in ROS production, and their expression is induced following the recognition of bacterial and fungal pathogens, particularly *AtRBOHD* and *AtRBOHF* (Torres et al., 2002; Torres et al., 2006). *Arabidopsis* serine/threonine kinase *OXIDATIVE SIGNAL-INDUCIBLE1* (*AtOXI1*) and zinc finger protein *AtZat12* are both marker genes for ROS signaling (Rentel et al., 2004; Miller et al., 2009). *Arabidopsis* CATALASE1 (AtCAT1) and CATALASE2 (AtCAT2), mainly located in peroxisome, detoxify H_2O_2 and are induced by abiotic

stresses (Mittler et al., 2004). Cytosolic ASCORBATE PEROXIDASE1 (AtAPX1) also can scavenge H_2O_2 (Mittler et al., 2004). To begin to understand their possible roles in *bik1*-mediated resistance to aphids, we performed quantitative PCR analysis to determine expression of these genes related to ROS production, signaling or scavenging (Fig. 2-5). Aphid infestation induced *AtRBOHD* and *AtRBOHF*, in both WT and *bik1* plants. However, elevated transcript levels of *AtRBOHD* and *AtRBOHF* were detected in *bik1* in the absence of aphids (Fig. 2-5A). The high basal expression could explain the faster and stronger ROS accumulation (Lei et al., 2014), agreeing with the bioassay result obtained by Miller et al (2009) where *rbohD* mutant was shown to be more susceptible to aphids. Similar expression pattern of *AtOXI1* and *AtZat12* (Fig. 2-5B) to the ROS-generating genes suggests that the ROS-responsive pathway is intact in *bik1* mutant. Conversely, no differential expression was observed between WT and *bik1* in ROS-scavenging genes; While *AtAPX1* was upregulated in both genotypes, *AtCAT1* and *AtCAT2* were repressed (Fig. 2-5C), possibly due to aphid-induced salicylic acid (Apel and Hirt, 2004). H_2O_2 can diffuse into plant cells to activate defense response including programmed cell death (Apel and Hirt, 2004). Enhanced ROS production coupled with rather inactive detoxification may have contributed to the heightened resistance in *bik1*, highlighting the importance of ROS in plant defense against aphids. On the other hand, excess ROS can eventually be harmful to the plant itself, which is reflected by the stunt growth and decreased fertility of *bik1* (Veronese et al., 2006; Lei et al., 2014).

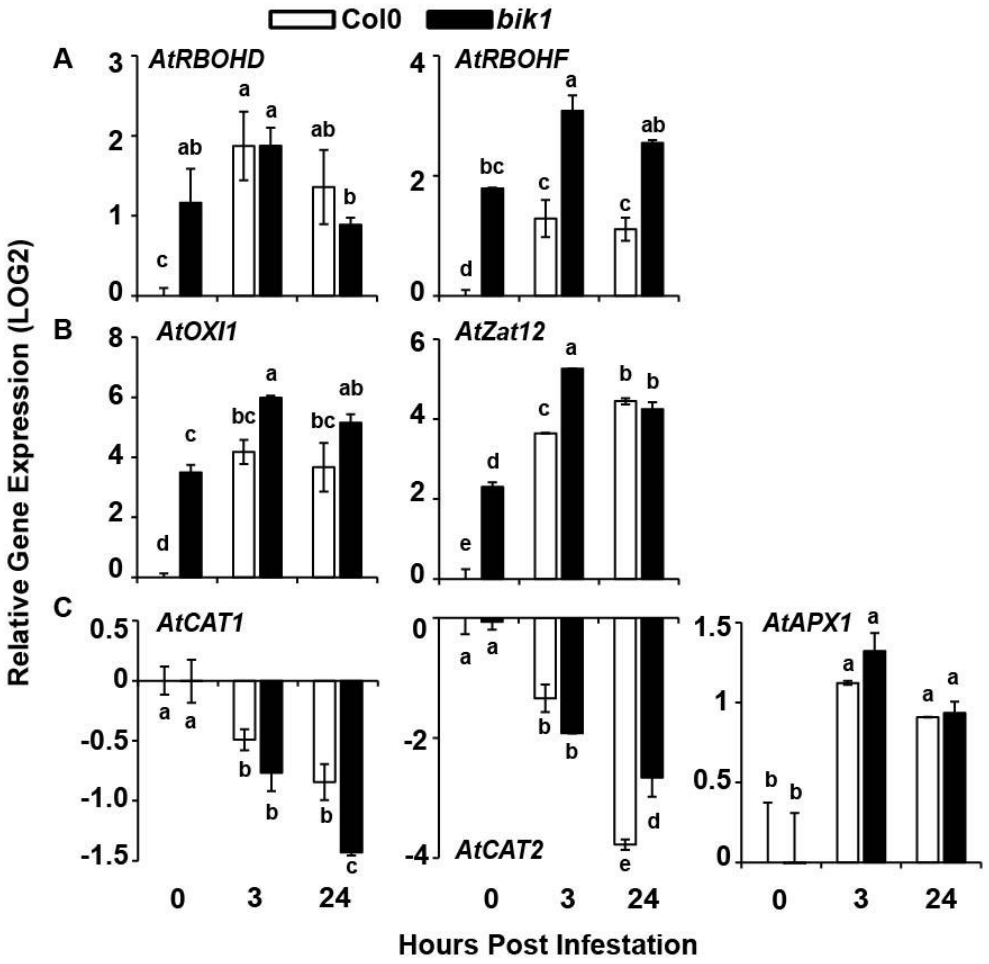

Figure 2-5. Elevated basal expression of ROS-generating **(A)** and ROS-responsive **(B)** genes in *bik1*, but no differential gene expression for ROS-detoxification genes **(C)**. Three-week-old plants were infested with 30 aphids for 0, 3 and 48 h. Total RNAs were extracted from *Arabidopsis* samples after removal of aphids, followed by reverse transcription and qPCR analyses. Gene expression levels at each time point were compared to the levels of the corresponding untreated Col-0 samples, which were arbitrarily set at 1. Transcript fold change is shown as LOG2-transformed bar graphs (means ± SE). Data were analyzed by one-way ANOVA. Duncan's multiple range test was used for pairwise comparisons. Means with different letters are statistically significant ($P < 0.05$). Each experiment was repeated at least 3 times. *AtRBOHD* and *AtRBOHF*, *RESPIRATORY BURST OXIDASE HOMOLOGUE D* and *F*; *AtOXI1*, *OXIDATIVE SIGNAL-INDUCIBLE1*; *AtZAT12*; *AtCAT1* and *AtCAT2*,*CATALASE1* and *2*; *AtAPX1*, *ASCORBATE PEROXIDASE1*.

2.3.4. *Detoxification enzyme activities are induced in aphid feeding on bik1*

Besides functioning as a signaling molecule in host plants to mediate defense gene activation, ROS may cause direct damage to insect tissues and cells (Bi and Felton, 1995; Apel and Hirt, 2004; Liu et al., 2010). Aphids perform poorly on *bik1* plants, but little is known whether they activate mechanisms to combat increased ROS in ingested plant materials. Comparison of catalytic activities of antioxidant enzymes in aphids fed on WT and *bik1* will shed some lights on how aphids respond to oxidative stress. Catalases (CATs) convert H_2O_2 into water and oxygen (DeJong et al., 2007). Glutathione S-transferases (GSTs) detoxify secondary oxidation products generated from ROS reacting with intracellular macromolecules (Hayes and Pulford, 1995). Levels of activity of these enzymes are believed to be crucial factors in determining the sensitivity of cells to broad spectrum of toxic chemicals including ROS. Here, we quantified enzymatic activities of CAT and GST by spectrophotometric-based enzymatic assays (Stumpf and Nauen, 2002; Weydert and Cullen, 2010). Compared to WT, aphids feeding on the *bik1* plants had significantly higher activities for the enzymes examined (Fig. 2-6). Results indicate that, as many other insect systems, aphids may also overproduce detoxification enzymes to fend off oxidative stress. The capability of coping with oxidative challenge in aphid is most likely accompanied by fitness cost, consistent with the retarded growth and reproduction.

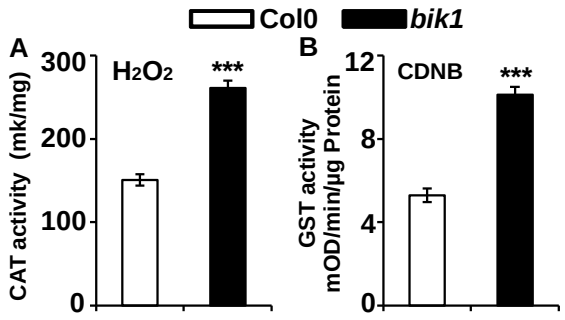

Figure 2-6. Detoxification enzyme activity in green peach aphids feeding on the Col-0 or *bik1* plants.
Age-synchronized aphid neonates were reared on 4-week-old plants for 7 days. 20 adult aphids were collected and homogenized with 0.2 M sodium phosphate buffer (pH 7.5). The supernatant was used for enzymatic assays. **(A)** Catalase (CAT) activity was analyzed by rate of H_2O_2 consumption at 240 nm. **(B)** Glutathione S-transferase (GST) activity was measured by using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate at 340 nm. All of the enzyme activities (means ± SE) were normalized by total protein determined by Bradford assay. Experiments were repeated at least three times. Data were analyzed by independent t-test. The asterisks indicate significant differences between samples ***($P < 0.001$).

2.3.5. *Aphids altered phytohormone contents and gene expression in bik1*

Aphid-induced plant defense and cell death pathways are often regulated by certain plant hormones (De Vos et al., 2005). To determine whether the resistance to aphids conferred by loss of BIK1 function involved defense-related plant hormones, we measured SA, JA, ET and ABA levels in the presence and absence of aphid feeding in both WT and *bik1* plants (Fig. 2-7A). Elevated basal SA (consistent with Veronese et al. (2006) and ET levels were detected in *bik1*, while JA and ABA contents were comparable in both genotypes. SA and ET levels increased in both WT and *bik1* upon aphid infestation, and the levels of both hormones were higher in *bik1* than in WT (Fig. 2-7A). No significant changes in JA and ABA were observed after aphid feeding. Basal

expression levels of the SA-signaling marker gene *PR-1*, and the ET/JA marker genes *ERF1* and *PDF1.2* were greater in *bik1* compared to WT (Fig. 2-7B). Aphid infestation upregulated expression of these genes in both WT and mutant plants. In comparison, basal expression of the JA-regulated transcription factor *MYC2* was similar in both genotypes and was not altered by aphid infestation in either genotype (Fig. 2-7B). These data imply that BIK1 may function as a negative regulator of SA and ET accumulation both in the presence and absence of aphid infestation, thereby suppressing expression of their responsive genes.

2.3.6. *Resistance to aphids conferred by loss of BIK1 function was SA-independent*

To assess the role that SA may play in *bik1* resistance to aphids, *bik1 sid2* and *bik1 nahG* plants were used for choice and no-choice tests (Fig. 2-8). Loss of *SALICYLIC ACID INDUCTION DEFICIENT2* (*SID2*) function blocks SA biosynthesis (Wildermuth et al., 2001), and *nahG* plants express salicylate hydroxylase that degrades SA to catechol (Delaney et al., 1994). In no-choice tests, the aphid numbers on *bik1 sid2* or *bik1 nahG* plants paralleled those on *bik1*, and numbers on SA-deficient *sid2* or *nahG* did not significantly differ from the WT (Fig. 2-8A). Similar results were obtained in choice tests (Fig. 2-8B), as well as from honeydew excretion assays (Fig. 2-8F). Apparently, reducing the SA level did not weaken aphid resistance in *bik1*, nor did it influence aphid response in WT. Therefore, elevated SA accumulation was not required for *bik1* resistance to the aphid, in contrast to its requirement for *bik1*'s resistance to a virulent strain of *Pseudomonas syringae* (Veronese et al., 2006).

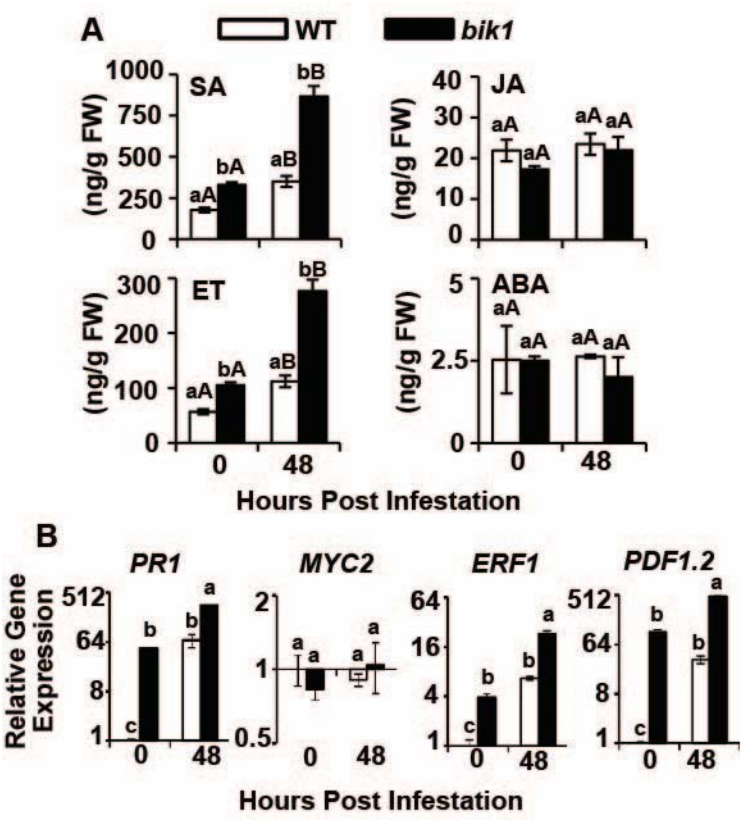

Figure 2-7. *bik1* shows higher basal and induced levels of SA and ET and elevated expression of their marker genes during aphid infestation than WT.
(A) SA, JA, ABA and ET levels in WT and *bik1* before and after aphid infestation. Three-week-old plants were infested with aphids for 48 h. Four replicates were used for each genotype. Hormone measurements were performed as described in Materials and Methods. Data were analyzed by independent samples t-test ($P < 0.05$). Different lowercase letters indicate significant differences between genotypes within the same treatment. Different uppercase letters indicate significant differences between treatments within the same genotype. **(B)** Relative expression of SA, JA and ET marker genes, *PR1*, *MYC2*, *ERF1* and *PDF1.2* in response to aphid feeding at 0 and 48 h time points. Data were analyzed by one-way ANOVA. Tukey's multiple range test analysis was used for pairwise comparisons of the difference between treatments for mean separation ($P < 0.05$).

To examine how SA impacted the aphid-triggered HR-like lesion formation, H_2O_2 production and cell death in *bik1*, DAB and trypan blue staining were conducted on the SA-deficient plants. No correlations were observed between the SA status and

lesion formation, H_2O_2 production or cell death phenotypes (Figs. 2-8C, 2-8D, 2-8E), a result supporting previous studies showing that SA is not essential for aphid defense in *Arabidopsis* (Pegadaraju et al., 2005). In contrast, a correlation was observed between resistance to aphids and H_2O_2 production as well as cell death occurrence. Notably, in terms of the plant size and morphology, *bik1 sid2* and *bik1 nahG* were closer to WT than to *bik1*, yet they exhibited levels of H_2O_2 production, cell death and aphid resistance comparable to *bik1*. Therefore, dwarfism was unlikely the cause of enhanced resistance to aphids in *bik1*. Heightened endogenous SA has been reported previously to confer *bik1* with resistance to the bacterial pathogen *Pst*DC3000 (Veronese et al., 2006). Results from our study revealed differential function of SA in BIK1-mediated plant responses to bacterial pathogens versus phloem sap-feeding aphids.

2.3.7. *Elevated ET signaling in bik1 increased aphid repellence during early stages of infestation*

Like SA, ET is known to play a key role in cell death and plant response to pathogens and insects (Dong et al., 2004; Cohn and Martin, 2005; Bouchez et al., 2007). To examine whether elevated ET has a role in aphid resistance in *bik1*, we pretreated plants with 1-methylcyclopropene (1-MCP), an inhibitor of ET action that binds to the ET receptor. In choice tests, there was no significant difference in the number of aphids on 1-MCP-treated *bik1* and WT plants 6 hr after aphid inoculation (Fig. 2-9), suggesting that 1-MCP may have compromised resistance in *bik1*. As time went on, however, 1-

MCP-treated *bik1* gradually regained their aphid repellence, presumably due to loss of 1-MCP function.

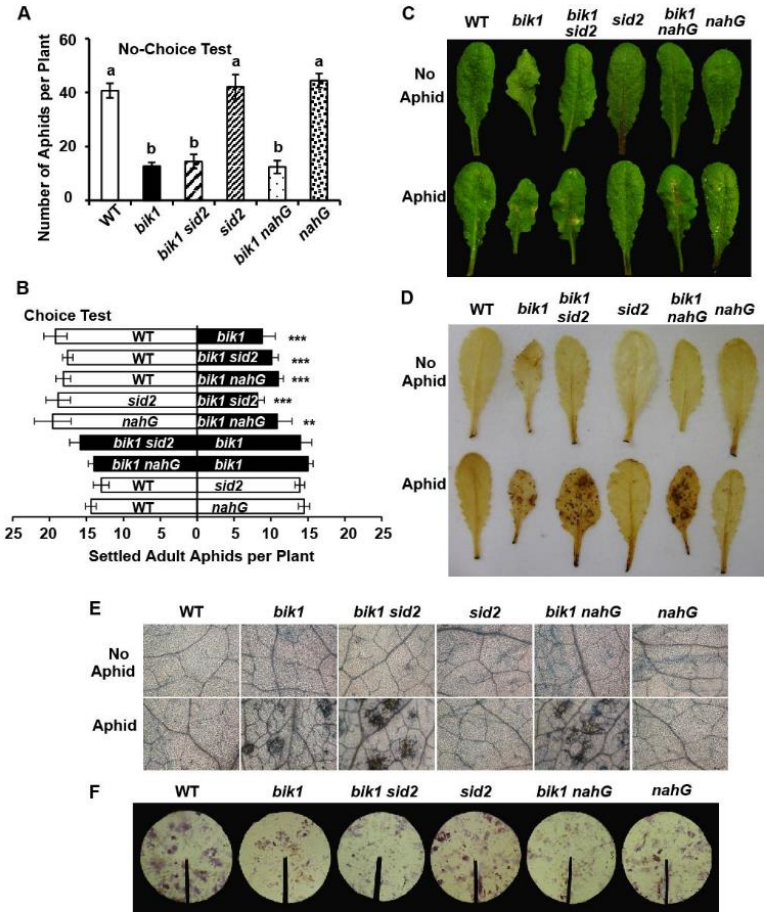

Figure 2-8. SA is not required for resistance to aphids and is not responsible for heightened hypersensitive response in *bik1*.
(A) No-choice and **(B)** Choice tests on genotypes indicated. **(C, D, E)** Representative leaf images of 4 to 5-week-old plants **(C)**, DAB staining (**D**, H_2O_2 indicator) and trypan blue staining (**E**, cell death indicator) before (top panel) or after aphid infestation (bottom panel). **(F)** Ninhydrin staining of honeydew after 48 h aphid feeding. All experiments were performed as described in in Materials and Methods. Bars represent means ± SE. Statistical significance for treatment effects is marked * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$). Means with different letters were significantly different ($P < 0.05$).

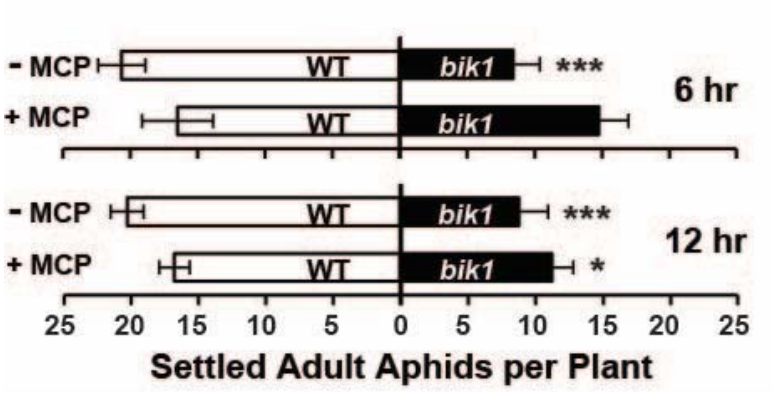

Figure 2-9. 1-MCP temporarily attenuates *bik1* deterrence of aphids.
Choice tests between 3-week-old WT and *bik1* plants in the presence and absence of 1-MCP. Settled aphids were recorded 6 and 12 h after aphid infestation. Application of 1-MCP began 5 days prior to choice tests, and was reapplied every 12 h to prevent the loss of its effectiveness. Control plants were subjected to the same manipulation without 1-MCP. Statistical significance for treatment effects is marked * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

Since the 1-MCP effect was temporary, this pharmacological approach was limited to choice tests. To further investigate whether increased ET contributes to *bik1* resistance to aphids, a genetic approach was used to impair ET signaling in *bik1* and longer-term no-choice tests were performed. The *bik1* mutant was crossed with two ET-insensitive mutants, *ethylene insensitive 2-1* (*ein2-1*) and *ein3-1* (Guo and Ecker, 2004; Broekaert et al., 2006). EIN2 (a transducer of ethylene signaling) and EIN3 (a primary ET-responsive transcription factor) are essential components of the ET signaling pathway. In no-choice tests, the *bik1 ein2-1* double mutant showed resistance comparable to *bik1* (Fig. 2-10A), suggesting that ET was not important in suppressing aphid reproduction in *bik1*, in agreement with honeydew secretion data (Fig. 2-10F). However in choice tests, blocking ET signaling in *bik1* (i.e. *bik1 ein2-1*) increased plant

attractiveness to aphids (Fig. 2-10B), implying that elevated ET in *bik1* contributed to its aphid repellence. Interestingly, when compared with *bik1*, *bik1 ein2-1* was preferred more by aphids early on. As experiments continued, the difference in the number of aphids on each genotype became non-significant. Thus, the overall effect of ET on *bik1*-mediated aphid resistance appeared to be only temporary and rather subtle.

The *bik1 ein2-1* double mutant maintained the small stature of the *bik1* single mutant (Fig. 2-1C). Feeding response in the *bik1 ein2-1* double mutant, i.e. lesion formation, H_2O_2 production and cell death upon aphid infestation, resembled those of *bik1* (Figs. 2-10C, 2-10D, 2-10E). Similar results were obtained with *bik1 ein3-1* plants (Fig. 2-11). Taken together, ET signaling in *bik1* was mainly involved in aphid deterrence initially in choice tests, but appeared to play little role in cell death-mediated defense in *bik1*.

2.3.8. *Aphid resistance and HR-like cell death in bik1 is PAD4-dependent*

PAD4 is a lipase-like protein that, upon aphid feeding, promotes premature leaf senescence to suppress insect reproduction and colonization (Pegadaraju et al., 2005; Pegadaraju et al., 2007). Aphids induced *PAD4* expression in both *bik1* and WT (Fig. 8A). Compared to the WT plants, *bik1* had much higher *PAD4* basal expression. Consistently, a senescence marker gene, *SENESCENCE ASSOCIATED GENE 13* (*SAG13*) regulated by *PAD4* during aphid infestation (Weaver et al., 1998; Pegadaraju et al., 2005) shared a similar expression pattern with *PAD4* (Fig. 2-12A). These results indicated that *BIK1* suppresses *PAD4* and senescence gene expression.

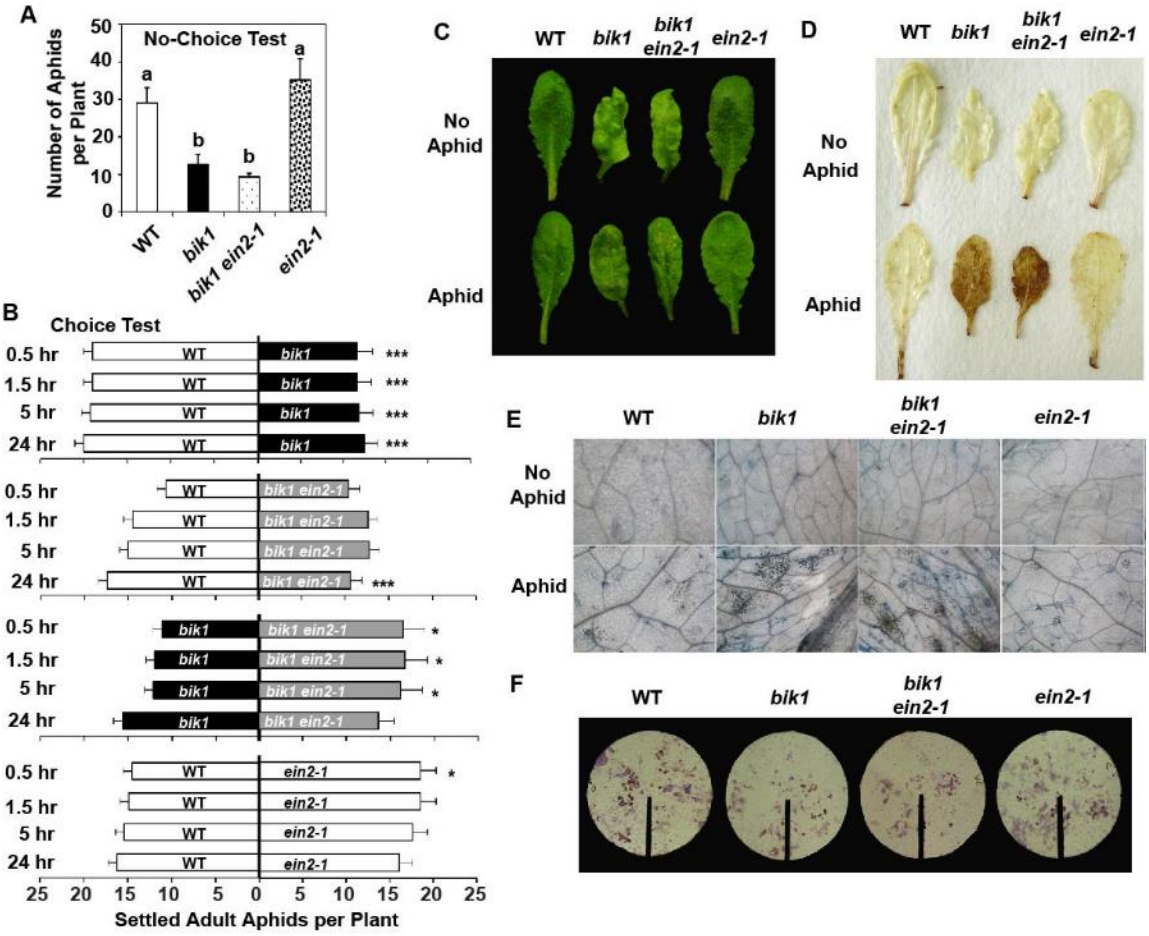

Figure 2-10. Elevated ET increases *bik1* repellence against aphids but shows no effect on aphid reproduction or on aphid-induced plant hypersensitive response.
(A) No-choice and **(B)** Choice tests on genotypes and at time points as indicated. **(C, D, E)** Representative leaf images of 4 to 5-week-old plants **(C)**, DAB staining (**D**, H_2O_2 indicator) and trypan blue staining (**E**, cell death indicator) before (top panel) or after aphid infestation (bottom panel). **(F)** Ninhydrin staining of honeydew after 48 h aphid feeding. All experiments were performed as described in in Materials and Methods. Bars represent means ± SE. Statistical significance for treatment effects is marked * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$). Means with different letters were significantly different ($P < 0.05$).

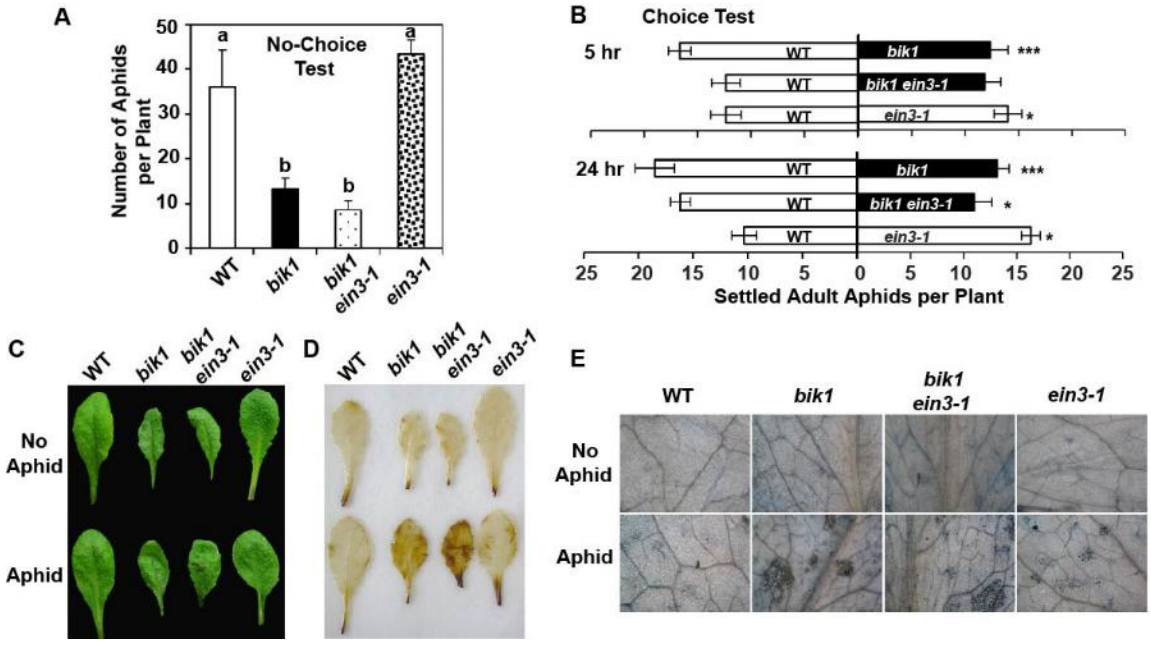

Figure 2-11. The effect of *ein3-1* mutation on *bik1*-mediated resistance against aphids. **(A)** No-choice and **(B)** Choice tests on genotypes and at time points as indicated. **(C, D, E)** Representative leaf images of 4 to 5-week-old plants **(C)**, DAB staining **(D)**, and trypan blue staining **(E)** before (top panel) or after aphid infestation (bottom panel). All experiments were performed as described in in Materials and Methods. Bars represent means ± SE. Statistical significance for treatment effects is marked * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$). Means with different letters were significantly different ($P < 0.05$).

To learn whether potential interactions exist between BIK1 and PAD4 in cell death-mediated aphid resistance, we examined aphid performance on the *bik1 pad4* double mutant. In no-choice tests, aphid numbers and body weight were both significantly higher on *bik1 pad4* than on *bik1* plants, and were comparable to WT (Figs. 2-12B, 2-12C). Honeydew excretion showed the same trend (Fig. 2-12H). Likewise, in choice tests, aphids showed a strong preference for *bik1 pad4* when paired with *bik1* (Fig. 2-12D). Apparently, the antibiosis and antixenosis observed in *bik1* diminished

when the *pad4* mutation was introduced. The *pad4* mutant did not support more aphid growth than the WT plant, although it attracted more aphids in the choice test. Therefore, the suppression of aphid performance in *bik1* was dependent on elevated basal *PAD4* expression.

Consistent with insect performance, *bik1 pad4* plants displayed phenotypes similar to those of WT in terms of lesion formation, H_2O_2 accumulation and cell death (Figs. 2-12E, 2-12F, 2-12G). Inactivation of PAD4 in *bik1* blocked the cell death, indicating that PAD4 was required for hypersensitivity and aphid resistance resulting from loss of BIK1 function.

Interestingly, ET emission decreased in *bik1 pad4* compared to *bik1*, both in the presence and absence of aphids (Fig. 2-13). This observation suggested that PAD4 may positively regulate ET accumulation.

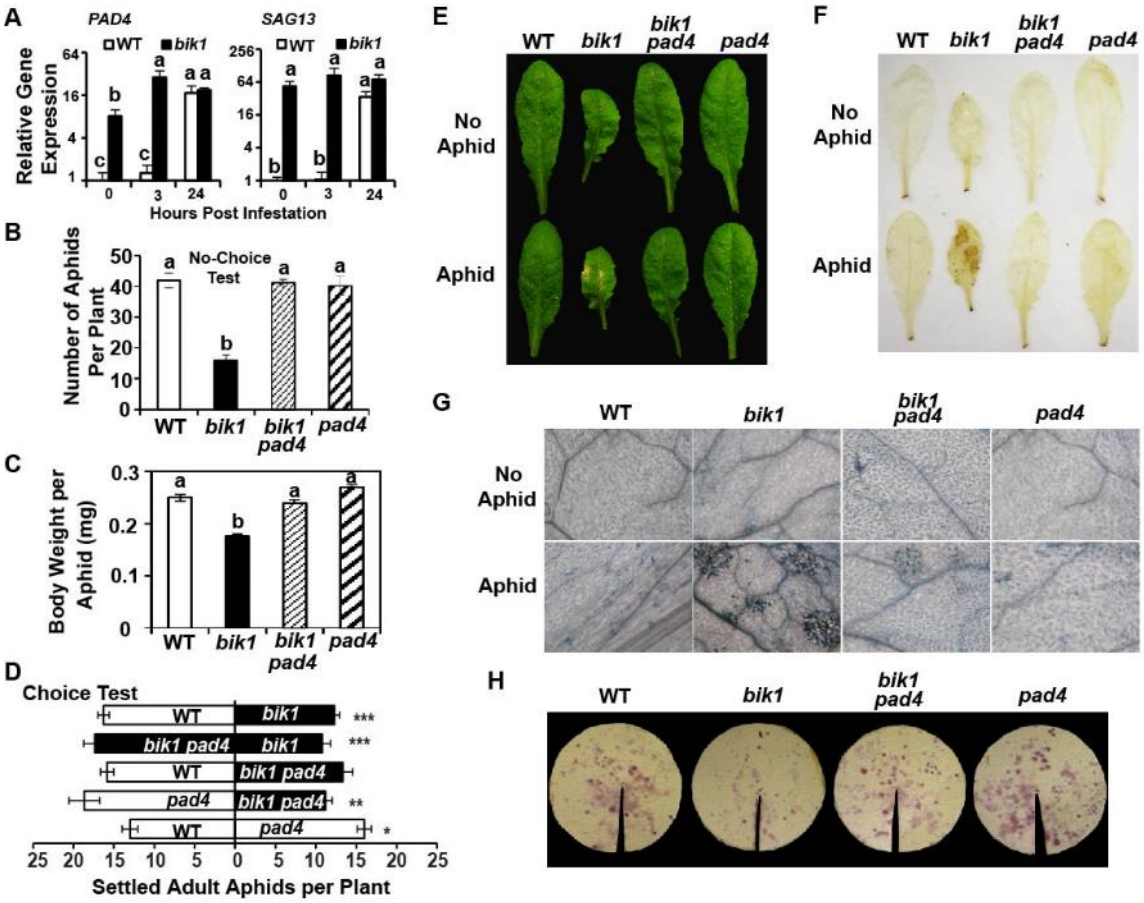

Figure 2-12. Resistance to aphids and aphid-induced hypersensitive response in *bik1* were *PAD4*-dependent.
(A) Relative expression of *PAD4* and *SAG13* in WT and *bik1* plants in the presence and absence of aphid infestation. Three-week-old plants were infested with aphids as described in Materials and Methods. **(B)** No-choice test, **(C)** average aphid body weight, and **(D)** choice tests were performed on genotypes indicated. **(E, F, G)** Representative leaf images of 4 to 5-week-old plants **(E)**, DAB staining (**F**, H_2O_2 indicator) and trypan blue staining (**G**, cell death indicator) before (top panel) or after aphid infestation (bottom panel). **(H)** Ninhydrin staining of honeydew after 48 h aphid feeding. Bars represent means ± SE. Statistical significance for treatment effects is marked ***(*P* < *0.05*), ****(*P* < *0.01*) or *****(*P* < *0.001*). Means with different letters were significantly different (*P* < *0.05*).

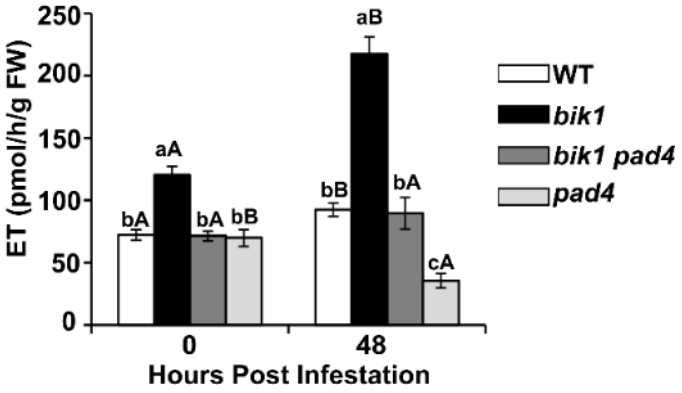

Figure 2-13. PAD4 promotes ET production.
ET production by WT, *bik1*, *bik1 pad4* and *pad4* plants measured before or after 48 h aphid infestation as described in Materials and Methods. Bars represent means ± SE from at least 6 individual plants. Different lowercase letters indicate significant differences between genotypes by one-way ANOVA and Tukey's multiple range test ($P < 0.05$). Different uppercase letters indicate significant differences between treatments by an independent samples t-test ($P < 0.05$).

2.3.9. *Loss of BIK1 function did not confer resistance to chewing insects*

Unlike aphids, chewing insects massively damage the host cells during infestation. To assess the role of BIK1 in *Arabidopsis* defense against chewing insects, we performed bioassays using fall armyworm (*Spodoptera frugiperda*) neonate larvae placed on 4-week-old WT and *bik1* plants (Fig. 2-14). No significant weight and size differences were detected between larvae reared on the two genotypes (Figs. 2-14A, B). In addition, fall armyworm elicited comparable H_2O_2 production on WT and *bik1* plants (Fig. 2-14C). The data suggested that BIK1 has distinct roles in *Arabidopsis* response to two groups of insects that differ in their feeding behaviors. This observation is also different from a previous study showing that TPK1b, the tomato homolog of BIK1,

enhances host plant resistance against tobacco hornworm (*Manduca sexta*) (Abuqamar et al., 2008).

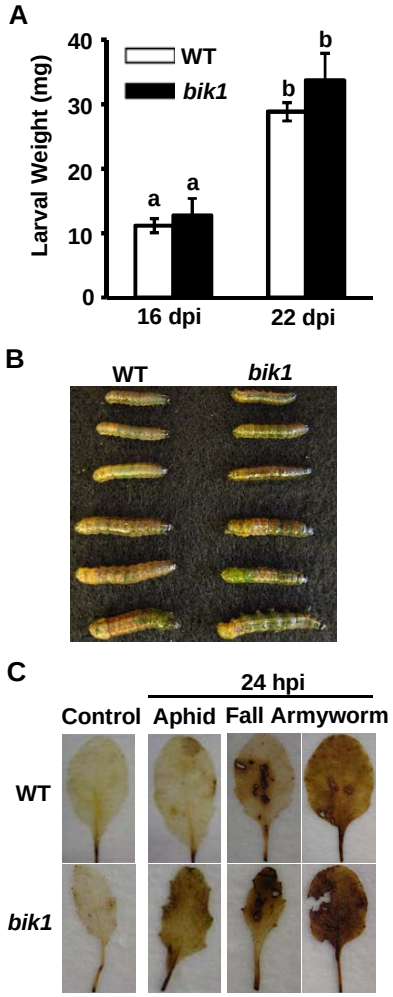

Figure 2-14. Loss of BIK1 function did not confer *Arabidopsis* resistance to fall armyworm.
(A) Comparison of larval body weight after 16 d or 22 d feeding on WT or *bik1* plants (n = 30). Different letters indicate significant differences between samples ($P < 0.05$). **(B)** Images of representative larvae feeding on each genotype. **(C)** Images of DAB-stained WT and *bik1* plants after aphid and fall armyworm feeding.

2.4. Discussion

Plants in the natural environment are constantly challenged by insect herbivory and pathogen infection. As a result, they have developed a plethora of sophisticated means to cope with diverse biotic stresses. Given the common features between plant responses to phloem sap-feeders and pathogens, we studied several PAMP/MAMP signal receptors for involvement in plant response to aphids using their loss-of-function lines. While FLS2, BAK1 and EFR did not seem to be associated with response to aphid infestation, BIK1 acted as a negative regulator of the defense response against aphids. This is in contrast to its positive role in resistance to fungal necrotrophs (Veronese et al., 2006) and flagellin-mediated immune responses (Lu et al., 2010). Thus, the PAMP-recognition components did not seem to have a parallel role in perceiving or transmitting signals from invading aphids.

2.4.1. *HR-like cell death could be pivotal for aphid resistance in bik1 plants*

The *bik1* mutant exhibited heightened resistance to aphids as well as enhanced local H_2O_2 production and necrotic cell death upon aphid infestation (Figs. 2-2 and 2-3). As in plant-microbe interactions, cell death could be either considered a plant defense factor, or viewed as an effect of aphid manipulation of host nutritional quality (Goggin, 2007). Although *bik1* plants displayed severe lesion formation, this aphid-induced symptom correlated with impeded aphid colonization, growth and reproduction. Thus, rather than a damage symptom, H_2O_2 accumulation and cell death represent a major defense mechanism in *bik1* to enhance resistance to aphids. These features were limited

to aphid-infested *bik1* leaves (Fig. 2-4) and unrelated to dwarfism (Figs. 2-1, 2-8). Furthermore, SA, JA, ET and ABA did not have major involvement.

Oxidative stress induced by insect feeding is believed to be an important component of plant resistance to invading insects. Detoxification of ROS may decrease antioxidant levels and increase toxic oxidation products in plants as shown in soybean following herbivory by *Helicoverpa zea* (Bi and Felton, 1995). In addition, increased H_2O_2 and other oxidative products in plants also directly damage the insect midgut and affect growth. Consumption of artificial diets containing even relatively low concentrations of H_2O_2 caused high mortality of insects (Liu et al., 2010). At high concentrations, ROS can react with almost all cellular macromolecules, including proteins, lipids and DNAs (Van Breusegem and Dat, 2006). Accordingly, the elevated ROS generated in *bik1* may result in decreased quantity and quality of nutrients and antioxidants, causing damage to aphid tissues and ultimately reducing their fitness. Furthermore, it is plausible that H_2O_2-potentiated HR in infected and adjacent cells could limit photoassimilate flow to the feeding sites, although it is questionable how effective such an approach can be, given that aphids can move away from their feeding sites before a sufficient defense response is mounted. Nevertheless, poor aphid performance on *bik1* plants relative to WT supported the hypothesis that rapid and potent HR-like cell death placed limitations on aphid infestation.

2.4.2. *ROS production, cell death and defense against aphids in bik1 required functional PAD4*

While loss of BIK1 function promoted aphid-induced lesions, no lesions were formed without aphid infestation (Figs. 2-3, 2-4). Furthermore, the spread of the aphid-induced lesions in *bik1* required continued aphid feeding (Data not shown). These data suggest that BIK1 does not directly repress but rather indirectly modulates a cell death pathway through an aphid-responsive component. We postulated that BIK1 may exert its negative regulation via PAD4, a lipase-like protein, for the following reasons: First, PAD4 regulates the activation of premature leaf senescence, i.e. a cell death-mediated resistance mechanism against aphids (Pegadaraju et al., 2005), consistent with the tight correlation between HR lesions and resistance we observed in *bik1*. Second, although PAD4 is involved in SA signaling, SA is not important for the defense against aphids conferred by PAD4, agreeing with our conclusion that *bik1* resistance is SA-independent. Third, expression of *PAD4* is induced in response to aphid feeding (Pegadaraju et al., 2005), potentially furnishing an aphid-triggered control point downstream of BIK1. Experimental results demonstrated that PAD4 was required for *bik1* resistance to aphids (Fig. 2-12). It should be noted that although more aphids preferred *pad4* plants over WT in the choice tests (Fig. 2-12D), no obvious increase in insect reproduction was observed on *pad4* in the no-choice tests (Fig. 2-12B). This is in contrast to the observations of Pegadaraju et al. (2005), who reported significantly higher population growth of *Green peach aphid* on *pad4* than on WT. Differences in plant growth conditions or in insect strain, age and quantity used by the two laboratories

could account for the different results. We witnessed relatively mild lesion formation in WT, which may explain the non-significant difference in aphid propagation on WT versus *pad4*. Furthermore, different conditions under which the ROS experiments were performed may explain the discrepancy in time needed for detection of ROS between different labs; in the current *in vivo* study, oral secretion was delivered via the aphid's fine mouthpart and was only in contact with a very limited number of plant cells, probably making ROS hard to detect in the early stage. Prince et al (2014), on the other hand, used leaf disks submerged in 5 mg/mL GPA-derived extract. It is possible that exposing the entire leaf tissue to a relatively high concentration of aphid elicitors permitted early ROS response. Alternatively, the early response could be triggered by factors in GPA-derived extract that normally would not come into direct contact with the host cells.

We propose that BIK1 modulates cell death and resistance to aphids through its control of PAD4 (Fig. 2-15). Removal of PAD4 function was sufficient to eliminate the strong HR-like cell death of *bik1* and restore its susceptibility to aphids. Ectopic expression of *PAD4* triggered more rapid cell death in aphid-infested leaves and stronger resistance to aphids than in WT (Pegadaraju et al., 2007). Inactivation of BIK1 repression in a sense resembles overexpression of PAD4. On the other hand, although aphid feeding induced *PAD4* expression and localized cell death in WT plants, DAB staining revealed only marginal differences in H_2O_2 production between the WT and the *pad4* mutant (Fig. 2-12). These data suggest that in WT plants, BIK1 suppression most likely is the dominant control factor for cell death, prevailing over the stimulus from

aphid feeding. It should be pointed out that high basal *PAD4* expression alone, i.e. in the *bik1* mutant without aphid feeding, was insufficient to result in cell death. Contrasting results of DAB staining of the *bik1* mutant with and without aphid treatment appeared to support this assumption. It is possible that PAD4-mediated cell death is initiated and propagated by aphid oral secretion-triggered signaling cascades, which are predominantly repressed by BIK1.

It should be noted that *bik1* is not the only mutant conferring PAD4-dependent aphid resistance. Loss of function of SUPPRESSOR OF SALICYLIC ACID INSENSITIVITY (SSI2), a desaturase, resulted in hyper-resistance to aphids, and the resistance required PAD4 as well (Louis et al., 2012). As with *bik1*, *ssi2* resistance diminished in the *ssi2 pad4* double mutant. But unlike the *bik1* mutant that expressed high basal *PAD4* transcript, the *ssi2* mutant did not show elevated *PAD4* expression in the absence of aphid feeding. Thus, the role of PAD4 in aphid resistance could be regulated by distinct pathways; while *bik1* may exert its resistance through releasing the suppression of PAD4 by BIK1, the interaction with SSI2 could be indirect.

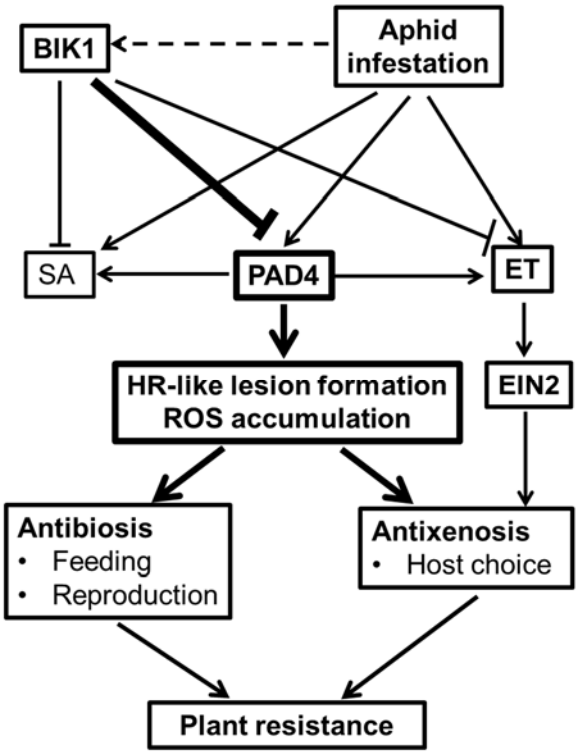

Figure 2-15. Model depicting *Arabidopsis* resistance to aphids conferred by *bik1* mutant.
PAD4 is a positive regulator of aphid-induced plant antibiotic and antixenotic responses. PAD4-regulated defense, potentially resulting from ROS-mediated cell death, is suppressed by BIK1. Based on the intensity of DAB staining, the BIK1 suppression is presumably much stronger than the aphid induction, illustrated by thicker lines in the graph. BIK1 also suppresses SA and ET accumulation. SA has no direct influence on resistance to aphids. ET increased host repellence early on, possibly prior to significant ROS production.

2.4.3. *Pleiotropic effects of BIK1*

It is rather counterintuitive, at first glance, that a gene like *BIK1* that confers plant susceptibility to invaders exists. A logical explanation could be that it plays an indispensable role in other processes, and/or is involved in multiple pathways in the plant where a balance has to be achieved through cross-talk. Constitutive defense is

often associated with fitness costs, e.g. altered leaf morphology, stunted growth and decreased fertility (Heil and Baldwin, 2002). Evidently, BIK1 is necessary for normal plant growth (Veronese et al., 2006) and seed production (Table 2-2). High levels of SA may be a major causal factor for the aberrant development and reduced growth of *bik1* since SA depletion by *sid2* and *nahG* largely restored the WT stature of *bik1* plants (Fig. 2-1; Fig. 2-8). Furthermore, the defect in SA accumulation in *pad4* could be responsible for the near WT plant form and leaf shape of the *bik1 pad4* double mutant (Fig. 2-1; Fig. 2-12). Indeed, many lesion mimic mutants display altered plant morphology due to production of elevated levels of SA and its constitutive interaction with other pathways (Lorrain et al., 2003). Therefore, it is very likely that BIK1 regulates normal plant growth in part by controlling SA levels. Conversely, *bik1 ein2-1* and *bik1 ein3-1* double mutants suffered the same growth suppression and aberrant development as the *bik1* single mutant, and did not show any phenotypic recovery (Figs. 2-1, 2-10, 2-11). Therefore, despite the essential role of ET in plant development, it is unlikely that the elevated ET level contributed to the *bik1* growth abnormality.

Notably, although BIK1 enhanced susceptibility to aphids, its presence did not block induction of effective aphid resistance genes but reduced their basal expression (Fig. 2-12). Perhaps, without BIK1 the penalty in general plant fitness imposed by maintaining a defense system in a no-pest environment outweighs an immediately available defense when plants are facing aphid attack. Besides plant development, BIK1 confers resistance to necrotrophic pathogens (Veronese et al., 2006) and is involved in activation of PAMP-triggered signaling pathways (Lu et al., 2010). Our current study

showcased the crosstalk among signaling pathways involved in plant development and defense against insects versus pathogens.

In contrast to our results showing that BIK1 negatively regulated resistance to a phloem sap feeder and had no effect on a chewing insect, studies on the BIK1 homolog in tomato, TPK1b, indicate that TPK1b positively regulates plant resistance against herbivory of tobacco hornworm, also a chewing insect (Abuqamar et al., 2008). Since TPK1b rescues the phenotype of the *Arabidopsis bik1* mutant, i.e. restoring its resistance to *Botrytis*, TPK1b and BIK1 are thought to perform similar functions in their respective species. The differential, even opposing functions exhibited by BIK1 and TPK1 suggests that the involvement of BIK1 in plant defense against insects could be shaped by specific insects through their distinct feeding styles and unique interactions with their host plants formed over the long history of coevolution.

Our study has drawn an important link between ROS production/cell death and plant resistance to aphids. However, uncoupling cell death from insect resistance has also been reported in studies with *Medicago truncatula* (Klingler et al., 2009). In these studies, it is clearly demonstrated that HR lesions are not required for resistance to the pea aphid (*Acyrthosiphon pisum*). In plant-pathogen interactions where the HR is often considered a major form of resistance, it has been shown that the *Arabidopsis* defense no death (*dnd*) mutant exhibits enhanced resistance against pathogen infection in the virtual absence of HR cell death (Yu et al., 1998). Further investigation is needed to establish whether the hypersensitivity is the basis for aphid resistance in *bik1* plants. It also

remains to be elucidated whether HR lesions directly cause plant defense or if they are the consequence of defensive biochemical reactions activated by aphids.

3. CIRCADIAN CLOCK-ASSOCIATED 1 MODULATES PLANT RESISTANCE AGAINST GREEN PEACH APHIDS

3.1. Introduction

The circadian clock, the endogenous oscillators found in most organisms, phases physiological, metabolic, and behavioral activities to specific time of day, to anticipate the dynamic day/night changes and confer fitness advantages (Dodd et al., 2005; McClung, 2006; Doherty and Kay, 2010; McClung and Gutierrez, 2010; Farre and Weise, 2012). Circadian clock comprises multiple interlocking feedback loops among clock components to generate 24-h oscillations (Pruneda-Paz and Kay, 2010; Staiger and Green, 2011; Bujdoso and Davis, 2013; Seo and Mas, 2014). In nature, plants are continuously challenged by pathogen invasion and insect herbivory, and have evolved a blend of strategies to counter specific biotic stresses. Constitutive activation of defense pathways is energetically costly. Therefore, it is reasonable to assume that synchronizing circadian regulation of defense pathways with external conditions can confer an advantage to the plants while minimizing energy consumption. Expression of genes responding to defense-related hormones, such as ABA, ACC, JA and SA, are gated by clock and exhibit phase-specific activation (Covington et al., 2008) In return, hormones serve as external cues to affect clock output (Hanano et al., 2006). The homeostasis of reactive oxygen species (ROS), the hallmark of plant stress responses, is regulated by CCA1 (Lai et al., 2012). Recently, increasing evidence has implicated direct involvement of the circadian clock in defense responses. Resistance to the

oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) and a chewing insect, *Trichoplusia ni* (cabbage looper) in *Arabidopsis* is clock-dependent, higher at dawn than at dusk (Wang et al., 2011; Goodspeed et al., 2012; Zhang et al., 2013). The effects of clock oscillations on plant defense against the bacterial pathogen *Pseudomonas syringae* vary by modes of invasion and strains. When infected by infiltration, like *Hpa* and *T. ni*, the plants at dawn are more resistance to both *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) and *P. syringae* pv. *maculicola* strain DG3 (*Psm* DG3) (Bhardwaj et al., 2011; Zhang et al., 2013). Conversely, spraying *Psm* DG3 at dawn promotes their propagation (Zhang et al., 2013). Surprisingly, the daytime-dependent defense responses to *P*sm with the avirulence gene *avrRpm1* (*Psm avrRpm1*) is irrelevant to clock (Griebel and Zeier, 2008). Furthermore, altered expression of clock genes also affect plant resistance. The *cca1* and *lhy* double mutant is more susceptible to *Hpa* and *Psm* DG3 (Wang et al., 2011; Zhang et al., 2013). Intriguingly, overexpressing *CCA1* renders plant more resistant to *Hpa*, but compromises defense against *Psm* DG3 and *T. ni* (Wang et al., 2011; Goodspeed et al., 2012; Zhang et al., 2013). These research suggest that CC A1 plays discrepant roles in plant defense, depending on the mode of pathogen infection and specific types of stress.

Plant resistance to insect herbivores can be defined as antibiosis, which negatively impacts insect development and reproduction, and / or antixenosis, which repels insects. Greens peach aphid (*Myzus persicae*) manipulate host plant metabolisms and resource reallocation to obtain more nutrients (Walling, 2008; Wilson et al., 2011). In return, aphid behavior is also influenced by host plant metabolites. Amounts and

sugar compositions of honeydew excreted by cotton aphid (*Aphis gossypii*) fluctuate during day/night cycles (Gomeza et al., 2006), revealing the potential effect of clock on aphid performance. However, the direct link between clock and plant defense against phloem-sap feeding insects has not yet been elucidated. In the present work, we demonstrate that the circadian clock coordinates aphid feeding behaviors and host plant transcriptional response. *Arabidopsis* antixenotic resistance against aphid is controlled by the clock. Notably, constitutive activation of *CCA1*, the core clock component, strongly enhances plants antibiosis and antixenosis toward aphids through positively regulating the biosynthesis of indolic glucosinolates (GLSs), a group of important anti-aphid secondary metabolites (Hopkins et al., 2009).

3.2. Materials and methods

3.2.1. *Plant materials and growth conditions*

A. thaliana was planted on LP5 potting medium (Sun Gro Horticulture, Bellevue, WA) in chambers set at 23ºC (day) /21ºC (night), 65% relative humidity (RH) and 12 h light/12 h dark (LD) photoperiod at a light density of 100 μMoles $m^{-2}s^{-1}$. Mutant lines were compared to the Wild-type (WT) plants from the same genetic background in each experiment. Mutant or overexpression lines in the Col-0 background were *CCA1-ox* (Wang and Tobin, 1998), *cca1-1* (SALK_067780), *ztl-1* (SALK_069091), *ztl-4* (SALK_012440) (Wang et al., 2011). Mutant line in Ws background were *cca1-11 lhy-21* (CS9380) (Hall et al., 2003).

3.2.2. *Aphid rearing*

Green peach aphids were maintained on cabbage in a chamber at 21ºC, 65% RH, and a photoperiod of 12L/12D (63 μMoles $m^{-2}s^{-1}$).

3.2.3. *Measurement of honeydew excretion*

To detect honeydew droplet produced by aphid, bromophenol blue, a pH indicator (below pH 3.0 is yellow and above pH 4.6 is blue) was used as described (Klingler et al., 1998). Whatman filter paper was stained by a 0.1% solution of bromophenol blue with 2.5 mM concentrated HCl (pH 2.6, yellow staining) and then air dried. Honeydew droplets fell onto the paper, turning it to blue color. Honeydew amounts were counted as the number of droplets in filter paper.

3.2.4. *Aphid artificial diet assays*

The aphid artificial diet used in honeydew excretion analysis was prepared as described before with minor modification (Dadd, 1967; Kim and Jander, 2007). The diet contains 15% sucrose and 20 amino acids (Alanine, 10 mM; Arginine, 16 mM; Asparagine, 42 mM; Aspartate, 10 mM; Cysteine, 3.3 mM; Glutamate, 10 mM; Glutamine, 10 mM; Glycine, 10 mM; Histidine, 10 mM; Isoleucine, 6 mM; Leucine, 6 mM; Lysine, 10 mM; Methionine, 5 mM; Phenylalanine, 3 mM; Proline, 7 mM; Serine, 10 mM; Threonine, 12 mM; Tyrosine, 2 mM; Tryptophan, 4 mM; Valine, 7 mM). The pH of the diet was adjusted to 7.0 with KOH. The diet was sterilized by passing through a 0.20-μm filter (CORNING, New York) with syringe, and then stored in -20ºC. Aphids

were reared in a 30 mL transparent cup covered by a sachet encapsulating 150 μL diet between two layers of stretched Parafilm. Twenty adult aphids were kept in each diet-containing cup for overnight (~16 h), and then removed. Total 20 to 30 neonates were produced and subjected to honeydew measurement under LD or constant light for three days.

3.2.5. *Aphid bioassays*

For no-choice tests, three-week-old plants were used. Plants were grown in a 12 cm pot for 10 days, and then transferred to 30 mL cups (one plant per cup). At least 30 plants were used for each experiment. To obtain age-synchronized aphids, three adult aphids were transferred to each plant and removed after 6 h. Only one neonate was kept in a single plant. To evaluate the development time of each instar, insects were checked every 12 h for 8 days. Longevity and reproduction were recorded every 24 h throughout the life span of each aphid. Choice tests were performed as previous described (Lei et al., 2014). All insect treatments and bioassays were performed at 24 ~ 25ºC, 50% RH under 12L/12D or constant light (LL) photocycles.

3.2.6. *Plant damage assays*

To assess resistance or susceptibility of *Arabidopsis* lines against aphids, the damage to plants caused by aphid was evaluated. Experiments were conducted under constant light. 2.5-week-old *CCA1-ox* line, 3-week-old *cca1-11 lhy-21*, 4-week-old *cca1-1*, *ztl-1* and *ztl-4*, as well as their own wild-type plants, were challenged by 10, 30,

and 40 adult aphids, respectively. Plant damage was scored on a scale of 0 to 9 (Table 3-1). The damage of each plant was visually assessed every day after aphid infestation.

Table. 3-1. Plant damage scales (chlorosis and necrotisis) used for evaluating effects of aphids on host plants.

Scale	Description
0	No visual plant damage
1	Plant appears healthy with some isolated chlorotic or necrotic spots
2	Up to 5% of total leaf area becomes chlorotic or necrotic
3	Up to 15% of total leaf area becomes chlorotic or necrotic
4	Up to 25% of total leaf area becomes chlorotic or necrotic
5	Up to 40% of total leaf area becomes chlorotic or necrotic
6	Up to 55% of total leaf area becomes chlorotic or necrotic
7	Up to 70% of total leaf area becomes chlorotic or necrotic
8	Up to 85% of total leaf area becomes chlorotic or necrotic
9	Plant death or no recovery possible

3.2.7. *Determination of chlorophyll content*

Total chlorophyll contents of *Arabidopsis* plants were extracted and determined as described before (Aksoy et al., 2013). Total shoot tissues were ground into powder in liquid nitrogen. 100 mg fresh tissues were extracted in 1 mL 80% (v/v). The supernatant was collected after centrifugation at 13000 g for 5 min at 4°C. Absorbance at 646.8 and 663.2 nm was measured by a spectrophotometer (Beckman, DU-640). 1 mL 80% (v/v) acetone was used as blank. Total chlorophyll content (chla + chlb) was calculated as (7.15 $A_{663.2}$ + 18.71 $A_{646.8}$)/1000/Fresh weights of leaves.

3.2.8. *Gene expression analysis by quantitative RT-PCR*

Arabidopsis lines were grown in soil under LD photoperiods as mentioned above. 18 d-old plants were transferred to LL condition for 24 h, and then were harvested every 4 h across 2.5 d. Samples were frozen in liquid nitrogen and stored in -80 ºC. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) followed by RNase-free DNase treatment (QIAGEN, Valencia, CA). cDNA was synthesized with 2 µg of RNA using random hexamer primers and SuperScript™ II Reverse Transcriptase (Invitrogen). Quantitative RT-PCR (qRT-PCR) was performed in a 10-µL reaction using SYBR Green Mastermix (BioRad, Hercules, CA). Primers were designed as described previously (Lei, et al. 2014). Primer sequences are provided in Table 3-2. For data normalization, *Arabidopsis* UBQ10 (AT4G05320) was served as an internal control. ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) was used for running qRT-PCR reaction. The mean fold change in gene expression was calculated as described previously (Zhu-Salzman et al., 2003).

Table 3-2. Sequence of primers used in this study.

Gene name	Accession number	Sense primer (5'→3')	Antisense primer (5'→3')
Quantitative RT-PCR			
CCA1	AT2G46830	GATCTGGTTATTAAGACTCGGAAGCCATATAC	GCCTCTTTCTCTACCTTGGAGA
CYP79B2	AT4G39950	AGTCTAGTCACGATATGTTTCTGG	GTCTCATCTCACTTCACCGTC
CYP79B3	AT2G22330	GTCAAGTCTCGGAATGTCGT	GAGAATCATCAAGAAGCAAAGGG
CYP83B1	AT4G31500	GCCATGATATTGGATATTGTTGTGCC	CACCTATCACACTCCTCACTTCGT
MYB34	AT5G60890	AAGGTGGATGGCGTACTCTC	TCGTCTTCTTCAGGACTAAACTC
Genotyping			
cca1-1 (SALK_067780)	AT2G46830	GCCCAAATAAGTTTAGGTCCA	ATCAACCTTCAATCTTCTGCC
cca1-11 CS9380)	AT2G46830	GGCAGAAGATTGAAGGTTGATT	GCTTGCGTTTGATGTCTCT
lhy-21 (CS9380)	AT1G01060	GTGAACAAGTATCCCTTACCA	GACTTCCTTCCACGAATCAG
ztl-1 (SALK_ 69091)	AT5G57360	ACAACACGGGTATTAGAGAC	GCCAAATATCAAGATTCTGCCT
ztl-4 (SALK_012440)	AT5G57360	GAAACCCAGGAGGAGTAGCA	AGAGAACAACAGAAACAAGCAC

3.2.9. *Glucosinolate measurement*

Glucosinolate extraction was prepared as described previously with minor modifications (Mewis et al., 2012). Lyophilized *Arabidopsis* samples (20 mg) were extracted with 4 mL of 70% methanol, at 80°C in a waterbath for 10 min with intermittent shaking. After cooling, the samples were sonicated for 15 sec on ice (Branson sonifier-250, 30% duty cycle, 3 output control), extracted at 80 ºC for 10 min and centrifuged for 10 min at 4,000 rpm. The clear supernatant was transferred to a new tube. The pellet was re-extracted at 80°C once with 2 mL 70% methanol, and the supernatants were combined. To quantify the GLS amounts, 1 μmole of sinigrin hydrate (SIGMA, St Louis, MO) was added into each sample during the first methanol extract as the internal standard. To desulfate GLSs, methanol extract were loaded on columns filled with 0.8 mL of DEAE Sephadex A-25 (GE Healthcare). 0.5 g DEAE Sephadex were rehydrated in 5 mL 2 M acetic acid. Columns were pre-treated twice with 1 ml of 6M imidazole solution in 30% formic acid and washed twice with 1 mL MQ water (18.3MQ). Load all the extracts into the columns. The columns were washed twice with 1 ml of 0.02M sodium acetate buffer (pH 4.0). 10 U of aryl sulfatase solution (Sigma-Aldrich, H-1 from Helix) dissolved in 0.02M sodium acetate buffer, pH 4.0 were added into each column and incubated at room temperature for 14 h (overnight). Desulfo GLS samples were eluted from columns with 1 ml of MQ water.

3.2.10. *Phytohormone quantification*

Phytohormone were extracted by 500 µl of extraction buffer containing 1-propanol/water/ HCl [2:1:0.002 v/v/v]). 10 µl of 5 µM deuterated internal standards were added into each sample: d-ABA ([$^2H_6$](+)-cis, trans-absisic acid), d-IAA([$^2H_5$] indole-3- acetic acid), and d-JA (2,4,4-d_3; acetyl-2,2-d_2 jasmonic acid) and d-SA (d6-salicylic acid). 100 mg (fresh weight) of grounded fresh tissue was used for each extraction. Samples were extracted at 4°C for 30 min under darkness with constant shaking. Dichloromethane (500 µl) was added to each extracts and agitated at 4°C for another 30 min under darkness. Samples were then centrifuged at 13,000 RPM for 5 min. Lower layer was transferred to a glass vial carefully. Extracts were dried under nitrogen gas stream, and then resuspended in methanol (150 µl). Samples were transferred to 1.5 ml microcentrifuge tubes and centrifuged at 14,000 for 2 min to get rid of any debris. Clear supernatant (100 µl) was transferred into an autosampler vial. 7 µl of sample was injected into LC- (-)-ESI-MS/MS. The hormones were separated by a mobile phase consisting of Solution A (0.05% acetic acid in water) and Solution B (0.05% acetic acid in acetonitrile) with a gradient consisting of (time- %B): 0.3- 1%, 2-45%, 5-100%, 8-100%, 9-1%, 11-stop, at the rate of 600 µl/min.

3.2.11. *Bioinformatics analyses*

Circadian microarray datasets of WT and *lux-3* mutant were obtained from the DIURNAL database (http://diurnal.mocklerlab.org/). Over-representation of circadian-regulated genes or mis-regulated genes in mutants of clock components among insect

responsive genes was determined using Fisher's exact test (http://research.microsoft.com/en-us/um/redmond/projects/mscompbio/fisherexacttest/). Cis-elements in the target gene promoters were identified by ATHENA (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl).

3.3. Results

3.3.1. *Aphid honeydew excretion exhibits diurnal rhythm and circadian rhythm*

Aphid intimately associates with the host plant metabolism, which dynamically respond to circadian clock, suggesting that aphid may have acquired a clock-responsive feeding behavior to adapt to their host. Honeydew excretion is an indicator of aphid feeding activity. Therefore, we hypothesized that honeydew excretion may exhibit time-of-day specific changes. Biological processes exhibit diurnal-rhythmic are not necessarily clock regulated. Only the rhythm that maintains under constant light or constant dark is considered to be controlled by the clock. To address this question, honeydew droplets were counted every 4 h from aphid reared on *Arabidopsis* wild-type (Col-0) plants under 12-h light/12-h dark (LD) or constant light (LL) photocycles. Honeydew excretion peaked at dusk (ZT12) and dipped at dawn (ZT0) in LD (Fig. 3-1A, left). The honeydew excretion patterns in LL coincided with those in LD (Fig. 3-1A, right), proving that this rhythmic behavior is controlled by clock. Aphid excreted similar amounts of honeydew during the day and night in both LD and LL (Fig. 3-1A).

Both host plant metabolic changes and aphid biological clock itself can contribute to rhythmic honeydew production. To rule out the influence of the host plant,

we examined honeydew production from aphids feeding on artificial diets. Similar to those on the host plant, honeydew excretion reached peak and trough at dusk and dawn, respectively, in both LD and LL (Fig. 3-1B). However, aphid excreted 30% more honeydew during the night (Fig. 3-1B). These data indicate that aphid feeding behavior is governed by their clock and it prefer to consume during the night. This is opposite to cabbage loopers, which prefers the day (Goodspeed et al., 2012). Though feeding activity of aphid on both host plants and artificial diets reached peak and trough at similar time, the levels of consumption were different, revealing that aphid coordinates feeing activity with its endogenous clock, as well as the changes of host plants.

3.3.2. *The transcription of aphid-responsive genes in Arabidopsis exhibits circadian rhythm*

Circadian clock regulates expression of a substantial fraction of genes in *Arabidopsis*, which are involved multiple pathways, including plant development, defense responses, as well as hormone biosynthesis and signaling (Covington et al., 2008). To determine whether the clock modulates the transcriptional networks responding to aphid infestation, we performed bioinformatics analysis on published data sets. DIURNAL is a database containing information about expression profile of over 20,000 *Arabidopsis* expressed genes during 24-h cycles, as well as their Pearson correlation coefficient (r) to clock models (Mockler et al. 2007). Aphid-responsive gene sets in *Arabidopsis* were obtained by published microarray analysis (De Vos, et al. 2005). According to DIURNAL database, 16.8% of expressed transcripts in *Arabidopsis*

are circadian regulated by using r>0.9 as cutoff. Remarkably, over 40% of aphid up- (P=4.5 × 10^{-74}) or down- (P=8.7 × 10^{-153}) regulated genes showed circadian fluctuation in mRNA abundance, while genes responding to *Frankliniella occidentalis* exhibited little or no enrichment (Fig. 3-2A). *Pieris rapae*-responsive genes also displayed circadian rhythmic, though they were not as significantly as those responding to aphid (Fig. 3-2A). Constant with previous study (Covington, et al. 2007), clock-regulated genes were evenly distributed during 24-h cycle (Fig. 3-3A). Interestingly, time-course expression profiles revealed that expression of aphid-responsive genes peaked at s specific time of the day (Figs. 3-2B, 3-3A). In contrast to peak expression of aphid up-regulated genes at dusk, aphid down-regulated genes peaked at dawn (Fig. 3-2B). These time-specific regulation is circadian dependent, since these fluctuations nearly diminished in a clock mutant, *lux-3*, in which expression of both *CCA1* and *LHY* are dramatically reduced (Hazen et al., 2005) (Fig. 3-3B).

We also found the significant correlation between degrees of induction by aphids and circadian rhythmicity. Fold induction was positively correlated with percentages of clock-responsiveness, meaning that the genes with higher fold changes induced by aphid are more likely controlled by the clock (Fig. 3-2C, black line). Over 60% of the genes highly induced by aphid (> 6 folds) were circadian responsive. This is two times of chances than those among low induction genes (2 to 3 folds). In addition, the mean relative amplitudes of the rhythms were also positively correlated with their fold induction by aphid (Fig. 3-2C, gray lines).

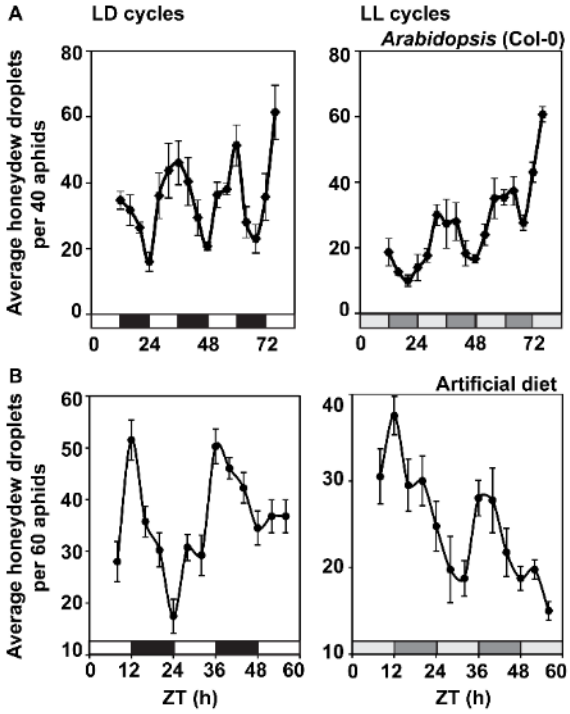

Figure 3-1. Honeydew excretion pattern of aphid is controlled by circadian clock and reaches peak levels during subjective dusk period.
Rectangles symbolize 12-h periods of day (white), night (black), subjective day (light gray), and subjective night (dark gray). Aphid entrained in 12-h LD cycles were transferred to 4-week-old *Arabidopsis* (Col-0) plant **(A)** or artificial diet **(B)**. Amounts of honeydew droplets were counted every 4 h under LD (left column) or LL (right column) conditions. **(A)** 8 second instar nymphs per plant, and 40 nymphs as a groups. Mean ± SE. N=3. Total is 120 nymphs. **(B)** 15 first instar nymphs were reared on each diet, and 60 nymphs were considered as a group. Mean ± SE. N=4. Total 240 nymphs were used. LD: light/dark. LL: constant light. ZT: zeitgeber time, time of the day.

To further investigate the role of clock components, we examined whether clock dysfunction plants affect expression of aphid-responsive genes. Both aphid-up and – down regulated genes were significantly enriched among mis-regulated genes in either *CCA1* over-expressing (*CCA1-ox*) plants or *cca1 lhy* plants (Fig. 3-2D). The percentages

of mis-regulated genes of *CCA1-ox* or *cca1 lhy* among aphid-responsive genes were around 3 times higher than those among the genome (Fig. 3-2D). Similar pattern were also observed among mis-regulated genes of *TOC1-ox*, *toc1-2* and *d975* (Fig 3-3C).

Altogether, these data suggest that the circadian clock regulates the *Arabidopsis* transcriptional network responding to aphid infestation, and led us to further examine the possible involvement of the biological clock, as well as its core regulator, such as CCA1, in plant defense against aphid.

3.3.3. *Arabidopsis exhibits temporal oscillations in resistance to aphids in choice test*

Previous studies show that *Arabidopsis* plants display circadian-dependent or independent time-of-day specific variations of resistance to several microbe pathogens and a chewing insect (Griebel and Zeier, 2008; Bhardwaj et al., 2011; Goodspeed et al., 2012; Zhang et al., 2013). Therefore, it is interesting to know whether plant endogenous clock has similar effect on aphid herbivory. To test this, we evaluated aphid performance on wild-type (Col-0) plants entrained either in-phase or out-of-phase with aphid (Fig. 3-4A). In order to access only circadian-controlled effect, all the plants were pre-treated in LL for 24 h and all experiments were conducted under LL and started at ZT1 (25 h after plants were exposed to LL). To determine plant antibiosis and antixenosis against aphid, both no-choice test and choice test were conducted, respectively. In no-choice test, the developmental times, average adult longevity, as well as total offspring production of aphids reared on in-phase plants were comparable to those on out-of-phase plants (Table 3-3). However, compared to in-phase plants, out-of-phase plants were more attractive to

aphids following 6 h after releasing of insects in choice test (Fig. 3-4B), revealing temporal variation in resistance to aphid infestation. Consistently, aphids feeding on out-of-phase plants excreted more honeydew, compared to those feeding on in-phase plants during the first 4 h of infestation (Fig. 3-4C).

To further determine whether functional clock is required for the in-phase resistance, plants with mutations in *CCA1* and *ZTL* were used for the choice tests as described above. As expected, preference for out-phase plants was abolished on *cca1*, *ztl1* and *ztl4* mutants with the dysfunctional clock (Fig. 3-4B). Taken together, these data demonstrate that *Arabidopsis* displays temporal oscillations in defense against aphids, mainly on antixenosis effect.

3.3.4. *CCA1, a core-clock regulator, is crucial in plant defense against green peach aphids*

We have established that host plant resistance against aphid responds to circadian clock (Fig. 3-4B), in agreement with clock's functions in plant defense against chewing insect and microbe pathogens. However, the biological functions of CCA1 often vary among interactions that occur between different organisms. Our bioinformatics analysis presented that aphid-responsive genes were highly enriched among transcriptomic profiles in *CCA1* mis-expressing plants (Fig. 3-2D). Thus, we postulated that CCA1 directly functions in plant resistance during aphid herbivory. To access this, both aphid behavior and plant damage symptoms were examined on *CCA1-ox* line, as well as mutants of *CCA1* and related genes, *LHY* and *ZTL* (Figs. 3-5, 3-6, 3-7).

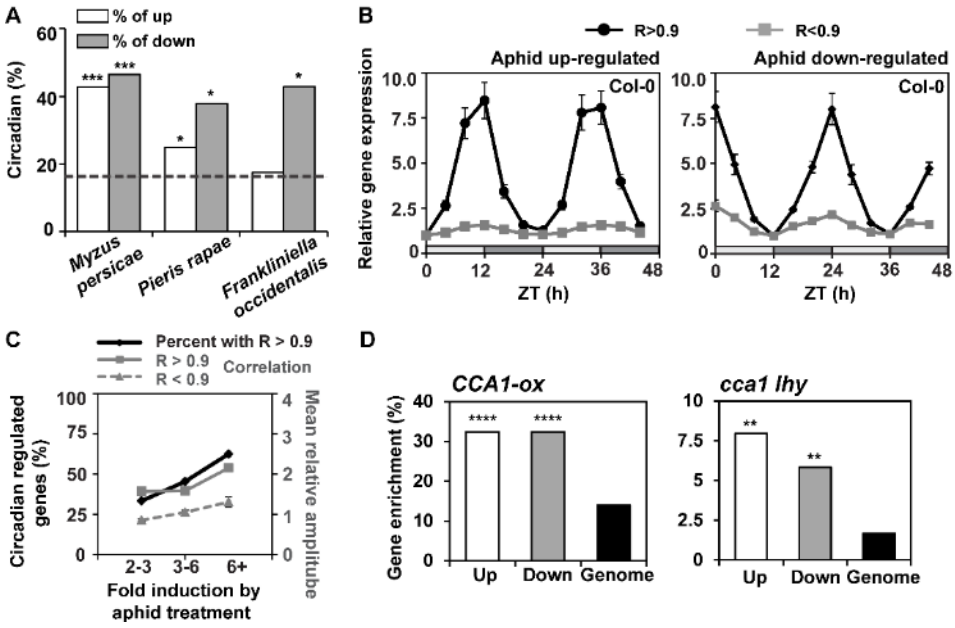

Figure 3-2. Circadian clock modulates transcription of aphid-responsive genes in *Arabidopsis* period.
(A) The percentage of clock-responsive genes among those up- (white bar) or down- (gray bar) regulated by different insects (*Myzus persicae*, *Pieris rapae*, and *Frankliniella occidentalis*). Data were analyzed by Fisher's exact test. Dashed line represents the percentage of clock-responsive genes among the whole genome. **(B)** Mean normalized microarray expression data for aphid-induced or repressed genes during different time of the day. Black line is average expression of circadian-regulated genes (circadian correlation $R > 0.9$), and gray line is for non-circadian genes. **(C)** Correlation between degree of induction by aphid and circadian regulation. Genes were classified into groups based on the degrees of induction by aphid (2-3, 3-6, and > 6 folds). Percentage of circadian-regulated genes is significantly correlated with fold induction by aphid (Black line, $r = 0.147$, $P < 10^{-4}$), and was plotted in the left y-axis. Both relative amplitude of circadian-regulated genes (gray solid line, $r = 0.290$, $P < 10^{-7}$) or noncircadian genes (gray dashed line, $r = 0.244$, $P < 10^{-7}$) are strongly correlated with fold induction by aphid, and were plotted in the right y-axis. **(D)** Percentage of mis-regulated genes in *CCA1-ox* or *cca1 lhy* plants at the whole-genome level (black bar), and among aphid up- (white bar) or down- (gray bar) regulated gene sets. Data were analyze///d by Fisher's exact test. Statistically significant circadian enrichment is marked as * $P < 0.01$, ** $P < 10^{-10}$, or ***, or $P < 10^{-50}$. Mean ± SE.

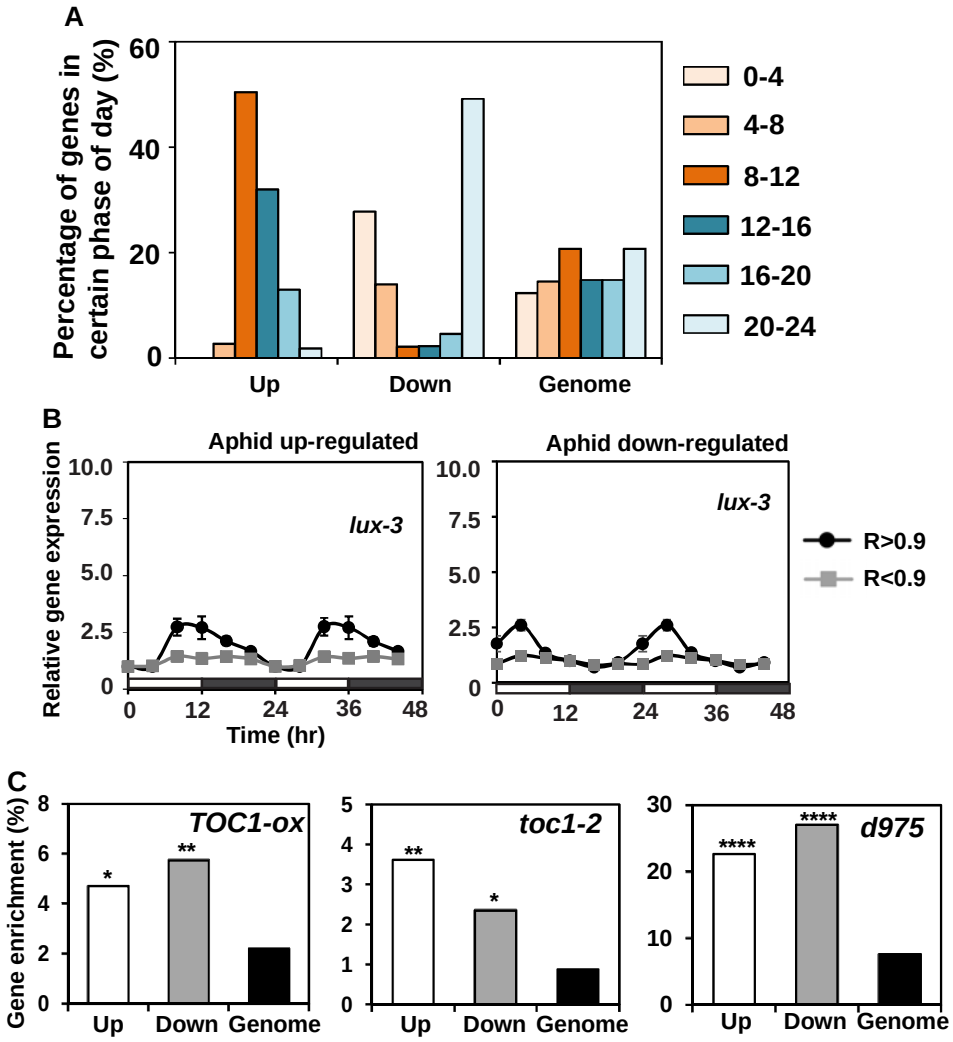

Figure 3-3. Expression of aphid-responsive genes in *Arabidopsis* are responsive to circadian clock.
(A) The phase distribution of aphid induced (up) and repressed (down) genes, as well as genes among the whole genome **(B)** Mean normalized microarray expression data for aphid-induced or repressed genes during different time of the day in *lux-2* mutant. Black line is average expression of circadian-regulated genes (circadian correlation $R > 0.9$), and gray line is for non-circadian genes. Circadian-regulated and non-circadian genes were defined based on their expression on Col-0. **(C)** Percentage of mis-regulated genes in *TOC1-ox*, *toc1-2* or *d975* plants at the whole-genome level (black bar), and among aphid up- (white bar) or down- (gray bar) regulated gene sets. Data were analyzed by Fisher's exact test. Statistically significant circadian enrichment is marked as * $P < 0.01$, ** $P < 10^{-10}$, or ***, or $P < 10^{-50}$. Mean ± SE.

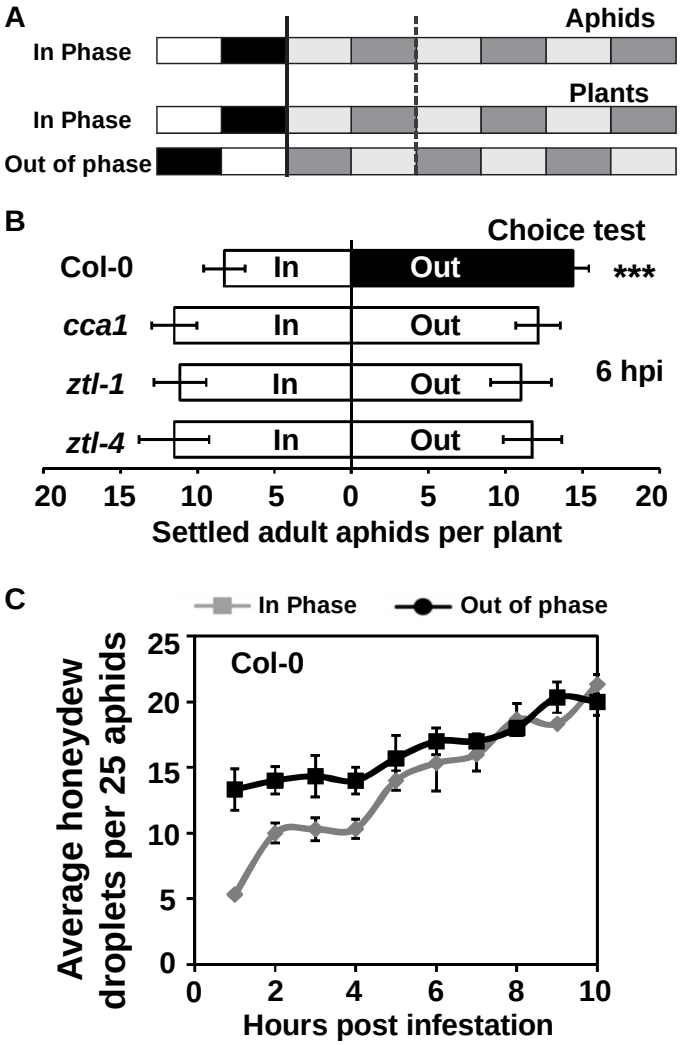

Figure 3-4. *Arabidopsis* entrained in-phase rather than out-of-phase with aphid entrainment is more resistant to aphid.
(A) Experimental design. Plants entrained in the same 12-h LD cycles as aphids were defined as in-phase plant; otherwise, those were out-of-phase. Solid line indicates the timing of transferring plant from LD to LL. Dashed line represents the timing of aphid infestation. **(B)** Choice tests. 3-week-old plants were used. 30 adult aphids were released between in-phase and out-phase plants of the same genotype. Settled insects per plant were counted after 6 h. Mean ± SE, N=8. hpi: hours post infestation. In: in-phase plant. Out: out-of-phase plant. **(C)** Average hourly honeydew production by aphid feeding on in-phase and out-of phase Col-0 plants. Age-synchronized 3rd instar nymph were used. Honeydew droplets were counted 1 h after releasing the insects. Mean ± SE, N=3

Table 3-3. Insect life table.

Genotype	Phase (in/out)	Developmental time (hours)					Adult longevity (days)	Total progeny amount
		1st	2nd	3rd	4th	Total		
WT (Col-0)	In	35.1 ± 1.4	27.9 ± 0.6	33.1 ± 0.1	43.8 ± 0.6	139.9 ± 2.2	18.7 ± 0.6 a	31.0 ± 0.9 b
	Out	35.4 ± 0.3	27.2 ± 1.3	31.3 ± 2.1	41.0 ± 0.2	135.0 ± 0.7	19.1 ± 0.1 a	28.5 ± 1.5 b
cca1	In	34.8 ± 0.6	25.8 ± 1.0	34.2 ± 1.6	42.7 ± 2.2	137.5 ± 2.8	18.6 ± 0.4 a	31.9 ± 1.4 b
	Out	31.7 ± 1.8	29.7 ± 2.4	31.3 ± 0.3	39.7 ± 0.3	132.3 ± 0.3	18.8 ± 0.7 a	28.9 ± 1.4 b
ztl-1	In	36.0 ± 0.1	30.1 ± 0.2	31.0 ± 0.3	41.8 ± 0.1	139.0 ± 0.2	17.3 ± 0.4 a	42.0 ± 0.4 a
	Out	34.8 ± 1.2	28.5 ± 0.4	32.9 ± 0.5	42.7 ± 1.0	138.9 ± 1.6	17.9 ± 0.5 a	40.2 ± 1.8 a
CCA1-ox	In	37.1 ± 0.6	32.4 ± 3.0	32.9 ± 0.9	44.2 ± 1.4	146.7 ± 0.5	17.5 ± 0.5 a	21.1 ± 1.1 c
	Out	37.1 ±.1.7	30.1 ± 2.3	32.6 ± 1.2	47.3 ± 1.0	147.1 ± 2.6	17.7 ± 0.3 a	18.9 ± 0.7 c

Intriguingly, honeydew production of aphid on *CCA1-ox* were merely half of those on Col-0 and became arrhythmic in both LD and LL (Figs. 3-1A, 3-5A, 3-6A), suggesting that *CCA1-ox* plants may confer constitutive resistance to aphid. In agreement with reduced feeding activity, aphids on *CCA1-ox* plants, in general, performed poorly in no choice tests compared to those on Col-0, including prolonged developmental time, less body weight, as well as reduced offspring production (Fig. 3-5B, C, D & E). In choice test, approximately one third as many aphids preferred *CCA1-ox* to Col-0 plants (Figs. 3-5F, 3-7A). Thus, over-expression of *CCA1* significantly enhanced both antibiosis and antixenosis against aphid. Similar experiments were also performed on *cca1*, *ztl-1*, *ztl-4*, and *cca1 lhy* mutants by comparing to their own wild type, Col-0 or Ws. No significant difference on antibiotic effect was detected, except *ztl-1* showed higher production of offspring compared to Col-0 (Fig. 35E). In contrast, mutations on these genes render plants more susceptible to aphids during choice tests (Figs. 3-5F, 3-7A). Though the effects on aphid performance in these mutants were not as dramatic as those in *CCA1-ox* plants, our results suggests that the expression levels of *CCA1* may be positively correlated with level of aphid resistance.

Consistent with poor aphid performance, *CCA1-ox* plants exhibited much less severe damage upon aphid infestation (Figs. 3-5G, 3-6). Col-0 plants started to develop damage symptom much earlier than *CCA1-ox* plants. *CCA1-ox* plants still stayed green 18 d after treatment, while Col-0 had already turned yellow 14 d after (Figs. 3-5G, 3-6B). Less chlorophyll and fresh weight were lost in *CCA1-ox* plants (Fig. 3-6C, D). Given their small stature, *CCA1-ox* plants resistance to aphid seems very remarkable.

Since it contains far less materials for aphid to feed on, the smaller plants are likely to be consumed earlier. In contrast to the overexpression line, *cca1*, *ztl-1*, *ztl-4*, and *cca1 lhy* plants wilted much faster, had greater loss of chlorophyll and fresh weights compared to WT plants (Figs. 3-5G, 3-7B, C, D, E).

Collectively, these results substantiate that CCA1 is an important positive regulator in plant resistance to aphid. In addition to the well-characterized role in the clock system, this study shows a novel function for CCA1 in the control of plant responses to aphid herbivory.

3.3.5. *Elevated enzymatic activity of glutathione S-transferase in aphid feeding on CCA1-ox plants*

The vigor of insect herbivore is determined by host plant quality, such as the type and amount of defensive metabolites (Awmack and Leather, 2002). To counter host plant toxins, insects have evolved multiple defense pathways, including up-regulation of detoxification enzyme activities (Despres et al., 2007; Li et al., 2007). Glutathione S-transferase (GSTs) detoxify a broad spectrum of substrates and play a key role during aphid adaptation to plant defensive metabolites, including glucosinolates (GLSs) (Francis et al., 2005). Therefore, if heightened defensive compounds in *CCA1-ox* plants account for the poor performance of aphids, GST activity should be up-regulated accordingly. Thus, GST activities from aphids feeding on *CCA1-ox* plants were measured and compared to those on Col-0 (Fig. 3-9A). GST activities of aphids, after 1.5 and 3.0-d feeding on *CCA1-ox* plants, were significantly higher than those on Col-0

(Fig. 3-9A). Similar pattern was observed among 2 generations of continuous rearing on Col-0 or *CCA1-ox* plants respectively (Fig. 3-9A). Elevated GST activities reveal a link between insect behavior and host plant resistant traits, and give us a hint that over-expressing *CCA1* gene may cause higher accumulation of anti-aphid compounds, such as GLSs, in *Arabidopsis*.

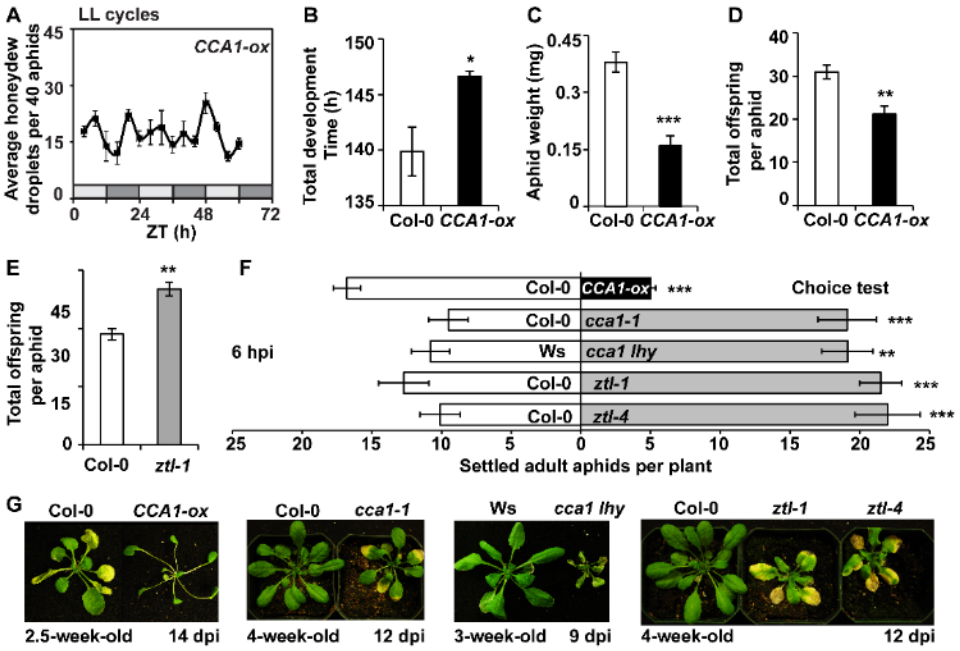

Figure 3-5. CCA1 is required for plant resistance to green peach aphid.
Plants were entrained in LD and then transferred to LL for 1 day before aphid treatments. **(A)** Average amounts of honeydew droplets of 40 insects feeding on *CCA1-ox* plants were recorded every 4 h. Total development time **(B)**, weight of 10-d-old adult insects **(C)**, and total number of offspring per insect during the whole life span **(D)** were compared between aphids reared on Col-0 or *CCA1-ox* plants. **(E)** Total numbers of offspring of insect feeding on Col-0 or *ztl-1* were counted. **(F)** Choice tests of indicated genotypes. **(G)** Representative images of plants after aphid infestation of genotypes indicated. Age of plant and infestation duration are indicated in the figure. Mean ± SE, N=3. Statistical significance for treatment effects is marked **P* < 0.05, ***P* < 0.01, or ****P* < 0.001. dpi: days post infestation.

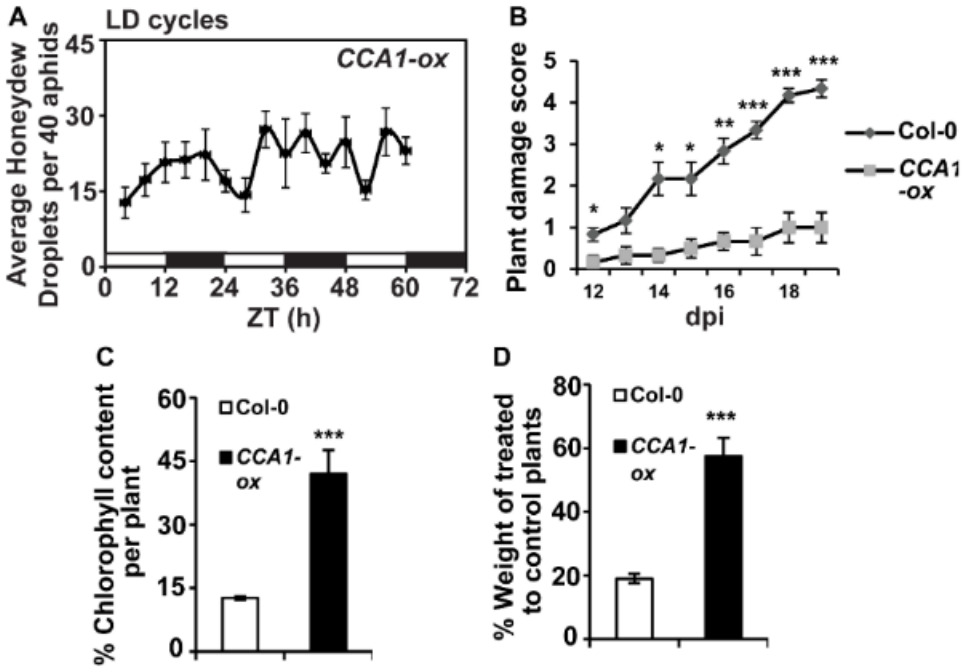

Figure 3-6. Overexpression *CCA1* enhances *Arabidopsis* resistance to aphid. Plants were entrained in 12-h LD and then transferred to LL for 1 day before aphid treatments. **(A)** Total amounts of honeydew droplets produced by aphid feeding on *CCA1-ox* plants were counted every 4 h under LL conditions. 8 second instar nymphs per plant, and 40 nymphs as a groups. Mean ± SE. N=3. Total is 120 nymphs. **(B)** Average plant damage score (0: no visual damage to 9: dead plant) for Col-0 and *CCA1-ox* plants over 19 days post aphid infestation. **(C)** Chlorophyll contents in plant infestated by aphid were measured and expressed as percentages of the chlorophyll content of the corresponding plants without aphid treatment. At least plants were used for each genotype. Experiments were repeat at least three times. **(D)** Percentage of weight loss of Col-0 and *CCA1-ox* plants caused by aphid. Data were expressed as percentages of the fresh weight of the corresponding plants without aphid treatment. Data represent means ± SE. At least 6 individual plants were used per genotype.

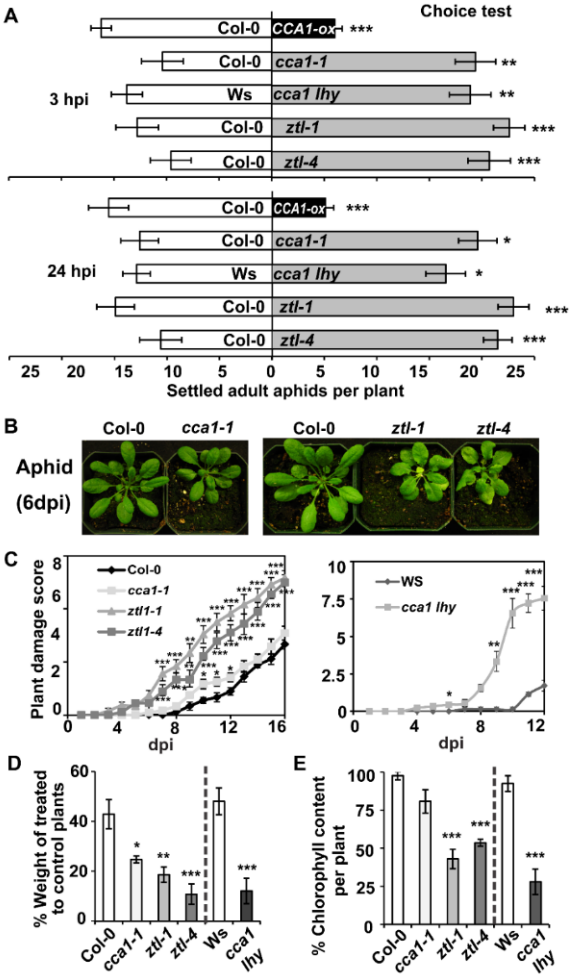

Figure 3-7. Mutations on clock-related genes render plant more susceptible to aphid infestation. Plants were entrained in LD and then transferred to LL for 1 day before aphid treatments.
(A) Choice tests of indicated genotypes. Insect were counted 3 and 24 hpi after releasing the insect. **(B)** Image of plant 6 days post infestation. **(C)** Average plant damage score (0: no visual damage to 9: dead plant) for wild-type and mutant plants over 19 days post aphid infestation. **(C)** Chlorophyll contents in plant infestated by aphid were measured and expressed as percentages of the chlorophyll content of the corresponding plants without aphid treatment. At least plants were used for each genotype. Experiments were repeat at least three times. **(D)** Percentage of weight loss of wild-type and mutant plants caused by aphid. Data were expressed as percentages of the fresh weight of the corresponding plants without aphid treatment. Data represent means ± SE. At least 6 individual plants were used per genotype.

3.3.6. *Higher expression of genes involved in indolic GLSs biosynthesis in CCA1-ox*

Glucosinolates (GLSs) are a large family of plant secondary metabolites grouped by the amino acids they are derived from, and target a various range of insect herbivores (Hopkins et al., 2009; Winde and Wittstock, 2011). Three major groups, including aliphatic, benzenic, and indolic GLSs, are found in plants (Sonderby, et al. 2010). Indolic GLSs, but not aliphatic GLSs, have antibiotic effects on aphids (Kim, et al. 2007 & 2008). During biosynthesis of indolic GLSs, CYP79B2/B3 are two cytochrome P450 enzymes that initiate the biosynthetic pathway by converting tryptophan to indo-3-acetaldoxime, the precursor of both indolic GLSs and indole-3-acetic acid (IAA) (Sonderby et al., 2010) (Table 3-4; Fig. 3-8). CYP83B1 controls a metabolic branch point by directing the flux of indo-3-acetaldoxime into indolic GLSs biosynthesis (Bak et al., 2001). *MYB34* encodes a Myb transcription factor that activates the expression of a tryptophan synthesis gene, as well as *CYP79B2*, *CYP79B3*, and *CYP83B1* (Celenza et al., 2005). The amounts of GLSs show circadian-rhythm in plants, including three members of indolic GLSs, Indol-3-ylmethyl (I3M), 4-methoxyindol-3-ylmethyl (4MO-I3M), and 1-methoxyindol-3-ylmethyl (1MO-I3M) (Goodspeed et al., 2013). Natural variation of GLSs amounts also affect the expression of components in the circadian clock system (Kerwin et al., 2011). Given that enhanced GST activity of aphid on *CCA1-ox* plants, we hypothesize that CCA1, as a transcription factor, regulates the time-of-day specific expression of indolic GLSs biosynthesis- related genes, thereby elevating the levels of indolic GLSs. To identify genes controlled by CCA1, we analyzed the

promoter regions of 9 genes involved biosynthesis of indolic GLSs by ATHENA program (O'Connor et al., 2005), as well as Circadian Correlation Coefficient (*r*) in DIURNAL. We found 5 out of 9 genes contain either CCA1-binding site and/or evening element (Table 3-4). To confirm whether these genes respond to the clock and are regulated by CCA1, we performed expression profiling on these genes in Col-0 and *CCA1-ox* plants in LL up to 52 h. Plants were entrained in LD first and released to LL 24 h before experiments. Of the 9 genes, *CYP79B2*/*B3*, *CYP83B1*, and *MYB34* displayed time-of-day specific expression in Col-0, supporting the notion that circadian clock control regulates the indolic GLSs biosynthesis at the transcriptional level. We observed that *CYP79B2*/*B3*, *CYP83B1*, and *MYB34* genes peaked around dusk (ZT12), and then gradually decreased until midday (ZT6) (Fig 3-9B, gray lines). The expression profiles of *CYP79B2*/*B3*, *CYP83B1*, and *MYB34* genes became arrhythmic in *CCA1-ox* (Fig 3-9B, black lines). In general, transcript expression in *CCA1-ox* were higher or similar to those in Col-0. Together, rhythmic oscillation of CCA1 is essential for clock-controlled time-of-day specific expression of indolic GLS biosynthesis genes.

Table 3-4. List of genes known to play a role in indolic GSL metabolism and regulation

Loci	Gene Name	Pathway	Evidence / biochemical & molecular functions	Phenotypes of mutants or over-expression lines	Reference	CCA1 binding site motif	EE motif	Circadian correl.
AT4G39950	*CYP79B2*	Biosynthesis of Indolic GLS	Biochem; converts Trp to indo-3-acetaldoxime (IAOx), a precursor to IAA and indole GLSs.	*cyp79b2/cyp79b3* double mutant is more susceptible to aphid	Kim, 2007	-818 -811 (-)	-192 -184 -	0.92
AT2G22330	*CYP79B3*	Biosynthesis of Indolic GLS	Biochem; Converts Trp to indole-3-acetaldoxime (IAOx), a precursor to IAA and indole GLSs	*cyp79b2/cyp79b3* double mutant is more susceptible to aphid	Kim, 2007	-790 -783 (-) -925 -918 (-)		0.94
AT4G31500	*CYP83B1*/*SUR2*	Biosynthesis of Indolic GLS	Biochem	*sur2* IG deficient mutant is overproduction of IAA	Barlier, 2000			0.76
AT1G24100	*UGT74B1*	Biosynthesis of Indolic & Aliphatic GLS	Biochem & genetic; Encodes a UDP-glucose:thiohydroximate S-glucosyltransferase, involved in GLS biosynthesis	*ugt74b1* mutant is overproduction of IAA	Grubb, 2005	-736 -729 + -584 -577 +		0.76
AT1G74100	*ATST5A*/*SOT16*	Biosynthesis of Indolic GLS	Biochem; Sulfotransferases catalyze the transfer of a sulfate group from PAPS		Klein, 2006 & 2008			0.91
AT2G20610	*SUR1*	Biosynthesis of Indolic & Aliphatic GLS	Genetic; Encodes a C-S lyase involved in converting S-alkylthiohydroximate to thiohydroximate in GLS biosynthesis.	*sur1*, overproduction of IAA	Mikkelsen, 2004			0.94
AT5G57220	*CYP81F2*	Biosynthesis of Indolic GLS	Biochem, genetic; catalyzes the conversion of indole-3-yl-methyl (13M) to 4-hydroxy-indole-3-ylmethyl GLS (4OH-13M)	mutant is more susceptible to aphids not lepidopteran	Pfalz, 2009			0.51
AT5G60890	*ATR1*/*MYB34*	Regulation of Indolic GLS	Genetic; transcriptional activation of indole GLS (IGS) biosynthetic genes	*atr1*D or *ATR*1 OE, activates Trp synthesis genes (*ASA1* and *TSB1*) and Trp-metabolizing genes (*CYP79B2*, *CYP79B3*, and *CYP83B1*)	Celenza, 2005	-990 -983 -		0.74
AT1G18570	*MYB51*/*HIG1*	Regulation of Indolic GLS	Genetic; transcriptional activation of indole GLS (IGS) biosynthetic genes	*HIG1-1D*, overproduction of IAA, activate IS biosynthesis gene	Gigolashvili 2007.		-698 -690 +	0.86

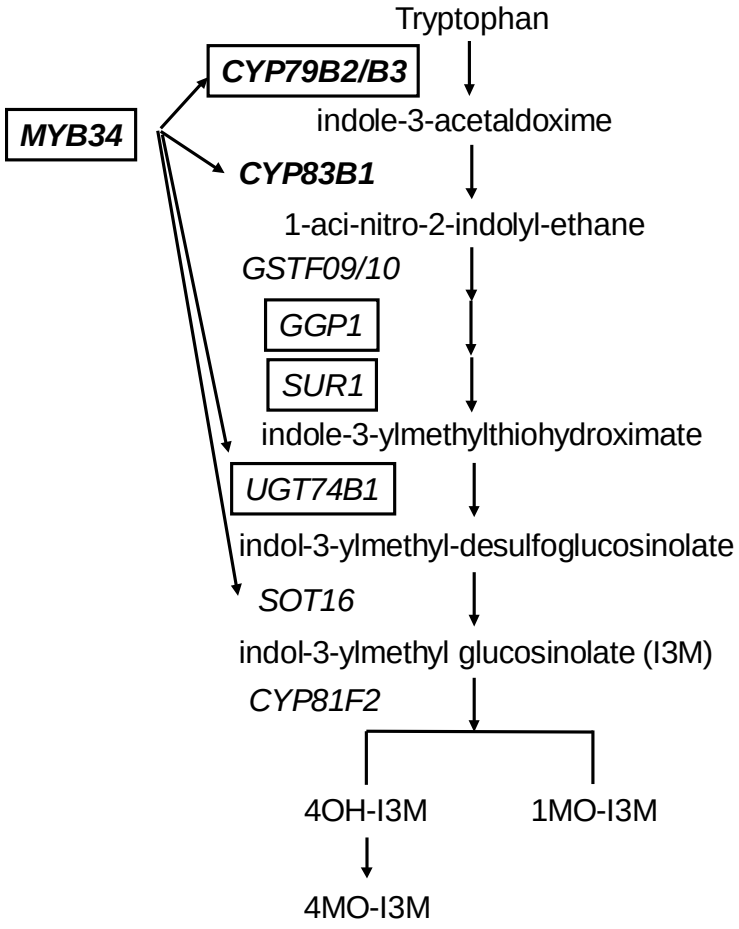

Figure 3-8. The biosynthesis pathway and transcriptional regulation of tryptophan-derived indolic glucosinolates in *Arabidopsis*.

To further support the control of indolic GLSs biosynthesis by CCA1, we also quantified GLSs in Col-0 and *CCA1-ox* plants before and after aphid infestation (Figs. 3-9C, 3-10). 4-week-old *Arabidopsis* plants were challenged by 30 aphids for 3 and 7 days in LL. Three indolic (I3M, 4MI3M and IMI3M) and 3 aliphatic (8MSOD, 4MTB and 4MSOB) GLSs were detected in our samples. Total amounts of indolic GLSs were significantly higher in *CCA1-ox* than in Col-0 with and without insect treatment,

whereas aliphatic GLSs amounts were comparable. Consistent with previous report (Kim and Jander, 2007), overall levels of GLSs, except 4MI3M and 1MI3M, were decreased 3 or 7 d after aphid feeding in Col-0. In *CCA1-ox* plants, total amount of indolic GLSs after 3-d aphid feeding maintained similar levels to control plants, and 4MI3M was significantly induced by aphid. Although the overall profile of aliphatic GLSs did not change by over-producing *CCA1*, 8MSOD increased in *CCA1-ox*. In agreement with gene expression profiles, *CCA1-ox* plants contain higher indolic GLSs, suggesting the regulatory role of CCA1 in indolic GLSs biosynthesis. Poor aphid performance on *CCA1-ox* plants most likely is due to elevated indolic GLSs.

3.3.7. *Jasmonate content was negatively regulated by CCA1*

Jasmonate (JA) and its derivatives, the key defense-related hormones in plant, are up-regulated in responses to wounding, chewing insect herbivory and pathogen infection. In *Arabidopsis*, JA is derived from α-linolenic acid and further metabolized into different derivatives. Currently, JA conjugated with amino acid isoleucine (JA-Ile) is the only known active form of JA. The accumulation of GLSs required JA-Ile-mediated signaling (Rasmann et al., 2012; Guo et al., 2013). Recent study indicates that JA content is controlled by the clock (Goodspeed et al., 2012). In this study, we found that CCA1 positively regulates the levels of indolic GLSs. This suggests that JA level is elevated in *CCA1-ox* line, resulting in increased indolic GLSs. Here, we quantified JA-Ile contents in Col-0 and *CCA1-ox* plants. Samples were harvested every 4 h during 24 h period. JA-Ile peaked around dawn in Col-0; while, its rhythmic pattern was lost in

CCA1-ox plants (Fig. 3-11). Remarkably, the levels of JA-Ile in *CCA1-ox* were more than 50% lower than those in Col-0. These data suggest that CCA1 regulates indolic GLSs biosynthesis in a JA-independent manner.

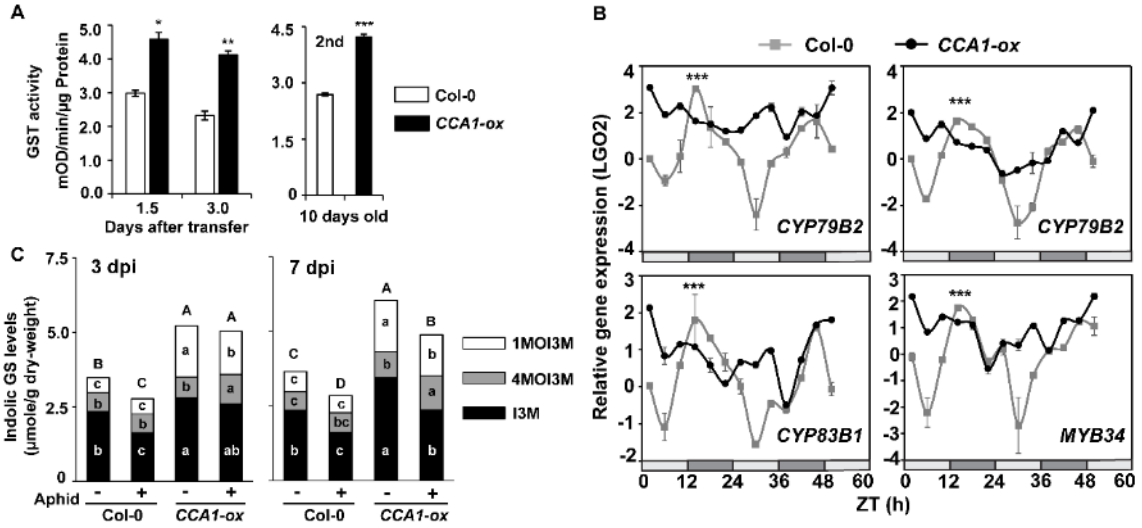

Figure 3-9. CCA1 promotes expression of indolic glucosinolate (GLS) biosynthesis-related genes and accumulation of indolic GLSs in *Arabidopsis*.
Plants were entrained in LD and then transferred to LL for 1 day before aphid treatments. **(A)** Enzyme activity of glutathione S-transferase (GST). Age-synchronized 2nd instar nymphs transferred to 4-week-old plants for 1.5 or 3 days (left), as well as 10-d-old adult aphids reared on Col-0 or *CCA1-ox* plants for 2 generation were subjected to enzymatic activity assays. Mean ± SE, N=6. **(B)** Expression profiles of 4 indolic GLS biosynthesis-related genes during 48 h in Col-0 (gray lines) and *CCA1-ox* (black lines) plants. Samples were collected every 4 h. Data were analyzed by One-way ANOVA (effect of time) (***$P < 0.001$). **(C)** Amounts of individual indolic GLSs in Col-0 or *CCA1-ox* plants before (-) and 3 or 7 d after (+) aphid infestation were quantified by HPLC. N=6. Data were analyzed by One-way ANOVA. Means with different letters were significantly different by Tukey's multiple range test ($P < 0.05$). Different lowercase letters indicate significant differences of individual indolic GLSs amounts among samples within the same time point. Different uppercase letters indicate significant differences of total indolic GLSs amounts among samples within the same time point. Statistical significance for treatment effects is marked *$P < 0.05$, **$P < 0.01$, or ***$P < 0.001$. dpi: days post infestation.

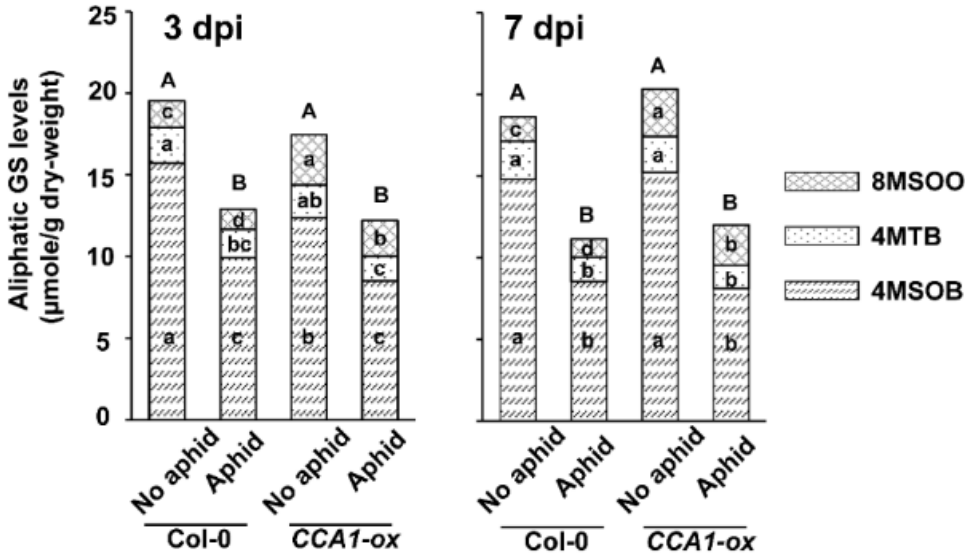

Figure 3-10. Total amounts of aliphatic GLSs are not significantly affected by overexpressing *CCA1* gene.
Amounts of individual aliphatic GLSs in Col-0 or *CCA1-ox* plants before (-) and 3 or 7 d after (+) aphid infestation were quantified by HPLC. N=6. Data were analyzed by One-way ANOVA. Means with different letters were significantly different by Tukey's multiple range test ($P < 0.05$). Different lowercase letters indicate significant differences of individual aliphatic GLSs amounts among samples within the same time point. Different uppercase letters indicate significant differences of total aliphatic GLSs amounts among samples within the same time point. Statistical significance for treatment effects is marked *$P < 0.05$, **$P < 0.01$, or ***$P < 0.001$. dpi: days post infestation.

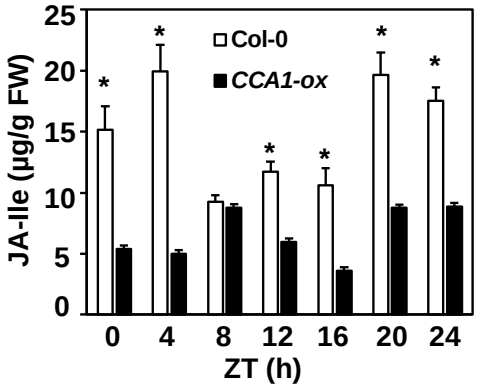

Figure 3-11. Total amounts of JA-Ile in Col-0 and *CCA1-ox* plants.
Plants were entrained in LD and then transferred to LL for 1 day before harvesting the tissues. Samples were collected every 4 h during 24 h period. Each data contains 6 biological repeats. The levels of JA-Ile were quantified by LC-MS. Statistically significant difference between Col-0 and *CCA1-ox* plants was analyzed by independent t-test is marked *$P < 0.05$.

3.4. Discussion

In higher plants, the circadian clock is the master regulator in various biological processes. The endogenous clock enable plants synchronize their physiological and metabolic activities with external conditions. Although previous studies reveal the functions of clock in plant defense against microbe pathogens and a chewing insect, there was no reports indicating its relationship with aphid defense. In the present work, we used bioinformatics, genetic and biochemical approaches to demonstrate that *CCA1* positively mediates plant resistance to aphids via modulating the levels of indolic glucosinolates, revealing the novel link between the circadian clock and plant defense response to aphid herbivory.

Aphid feeding behavior is governed by its endogenous internal clock and intricately coordinates with temporal oscillations of plant defensive metabolites. We found that aphids on artificial diets feed primarily during subjective night and peak their activity at dusk (Fig. 3-1). This feeding behavior is predominantly controlled by its clock, since the contents in artificial diets is relatively constant. However, aphid behavior is far more complicated in whole plant systems with the combination effects of host plant nutrients and defensive metabolites. Though aphid feeding on *Arabidopsis* still peaked at dusk, the overall feeding amounts were comparable during the day and night (Fig. 3-1). The differential responses to the artificial diet and host plant are very likely due to rhythmic variations in phloem sap during subjective day and subjective night. In *Arabidopsis*, the contents of amino acids, the essential nutrients aphid obtains from phloem sap, reach peak at subjective dusk (Espinoza et al., 2010). Moreover, the circadian accumulation of several well-known anti-aphid compounds, including JA and indolic GLSs, peak at midsubjective day, and decline quickly during subjective dusk (Goodspeed et al., 2012; Goodspeed et al., 2013). The insect clock-mediated aphid behavior is therefore advantageous, since it enable aphid to ingeniously avoid host plant defense and obtain nutrients with maximum efficiency.

Aphid-responsive genes exhibit clock-dependent rhythmic expression patterns in *Arabidopsis*, correlating with the timing of aphid feeding patterns (Fig. 3-2). Our bioinformatic analysis shows that genes induced by aphid peaked around subjective dusk; while, genes repressed by aphid peaked around subjective dawn. These patterns are in tune with the changes of aphid feeding activity, indicating that plant may

'anticipate' aphid feeding peaks at dusk. The scenarios that plants 'anticipate' biotic challenges according to a clock schedule give advantage to plant survival during microbe pathogen infection and chewing insect herbivory. However, given the nature of aphid infestation, the circadian oscillations of defense pathways may have limited effect on aphid population. It has been long known that aphids can 'deceive' their host plants by 'disguising' themselves to avoid effective defenses (Walling, 2008). Aphids strongly induce salicylic acid biosynthesis and signaling (Lei et al., 2014). Conversely, JA signaling or related metabolic products, such indolic GLSs are largely repressed or no changed by aphid treatment (Zhu-Salzman et al., 2004; Kim and Jander, 2007; Lei et al., 2014). Pharmaceutical and genetic experiments demonstrate that JA, other than SA, are the effective anti-aphid compounds (Zhu-Salzman et al., 2004; Pegadaraju et al., 2005; Lei et al., 2014). Further analysis of the transcriptomic data reveals that SA-responsive genes are significantly enriched among genes up-regulated by aphid and peak at dusk. Meanwhile, opposite pattern is observed in JA-responsive genes (Fig. 3-12). This finding suggests that although plant may 'anticipate' the peak period of aphid feeding and up-regulate aphid-inducible genes accordingly, it may not be sufficient to fight off aphid since SA is not an effective anti-aphid compounds.

Unlike chewing insects or some microbe pathogens, in-phase plants didn't display substantial increase of resistance to aphid. Since we started insect treatment in the early morning, in-phase and out-of-phase plants were in subjective morning and evening, respectively, at initial stage of infestation. Higher levels of clock-mediated JA, as well as indolic GLSs, during subjective morning, could account for aphid deterrence

observed in the in-phase plants. However, the advantage given to in-phase plants by enhanced antixenosis should be short-lasting, since the changes of in-phase or out-of-phase didn't significantly impact aphid population growth, the major contributor to crop damage. This is in agreement with our observation that although it took less time for aphid to settle on out-of-phase plants, insects reared on plants of either phase showed similar feeding activity eventually (Fig. 3-4). Admittedly, the efficiencies of temporal control of defensive compounds to fight off pests or pathogens are heavily dependent on the specific modes of infection or herbivory. As we discussed above, aphid can 'avoid' host plant clock-'scheduled' defense responses by reaching their feeding peaks at dusk when the defensive compounds greatly decrease and nutrients are still ample. Hence, this adept strategy enable aphid to maintain adequate feeding on either in-phase or out-of-phase plants.

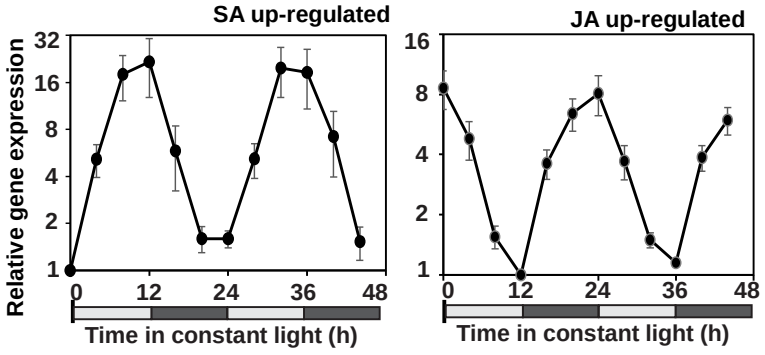

Figure 3-12. Mean normalized microarray expression data for SA-responsive genes among aphid-induced genes (Left) and JA-responsive genes among aphid-repressed genes (right) during different time of the day.

CCA1 is vital for plant defense against aphids and has distinct functions among different biological systems. Aphid perform poorly in *CCA1-ox* plants. In addition, the loss of CCA1, and related clock components, such as LHY and ZTL, impair plant resistance to aphids (Fig. 3-5). The phenotypes of *CCA1* mis-expressing plants establish a direct or indirect positive role of CCA1 during plant response to aphid infestation. Our cis-element analysis suggests that the CCA1 may directly target the promoters of a number of genes in the indolic GLSs biosynthesis pathway (Table 3-4). Further qPCR analysis confirms that CCA1 positively regulates the expression of indolic GLSs biosynthesis-related genes (Fig. 3-9). In agreement with transcript levels, hyper-accumulation of indolic GLSs was also detected in *CCA1-ox*. Overexpression of *CCA1* appears to enable the host plant to fight against aphid effectively by constantly activation of indolic GLSs biosynthesis pathways. In contrast, *CCA1-ox* plant is more susceptible to *T. ni* (Goodspeed et al., 2012). The discrepancy between the two systems may be due to differential effects of indolic and aliphatic GLSs on insect herbivores. Aliphatic, other than indolic, GLSs exhibits antibiotic effect on *T. ni* (Rasmann et al., 2012). Moreover, the substantial reduction of JA levels in *CCA1-ox* plants may render plant more susceptible to *T. ni* (Fig. 3-11).

CCA1 plays a central role in balancing growth and defense in plant. Disrupted rhythmic expression of CCA1 could manifest dramatic changes in various traits, including plant morphology, flowing time, metabolic profiles, as well as responses to stresses (Fukushima et al., 2009). Through overexpressing CCA1 renders plant more resistant to aphid herbivory, normal plant growth is notably hindered. *CCA1-ox* plants

display slow growth, reduced biomass, prolonged flowering time. Constitutive expression of defense pathways may confer competitive advantage in the presence of stresses, but it is also energy-costly, which affects the normal plant growth. Therefore, regulation according to the circadian clock is critical for balancing defense and growth, enhancing host fitness in nature environment.

In summary, our results showed the dynamic interaction between aphid feeding behavior and circadian clock-mediated defense response, which may have coevolved during long-time adaptation, thus raising the complexity of plant-insect interactions. We demonstrated that CCA1, the circadian clock core component, is a central regulator of host plant resistance to aphid by temporal control of defensive compounds, revealing a new interface between the plant host and aphid herbivores. Importantly, our study also established a novel molecular link between the CCA1 and indolic GLSs biosynthesis.

4. CONCLUSIONS

In this study, I have successfully demonstrated the roles of hypersensitive response as well as circadian clock in plant resistance to green peach aphids. In my first project, I found that BIK1 is a negative regulator during Arabidopsis defense against aphid by suppression of PAD4 dependent cell death. In wild-type plants, BIK1 predominantly represses aphid-induced hypersensitive response to maintain normal plant growth and development. Elevated salicylic acid and ethylene contents in *bik1* did not contribute to the HR-like symptoms. My Second project is to demonstrate the roles of the circadian clock in Arabidopsis resistance to green peach aphid. Wild type Arabidopsis plants exhibit temporal oscillation of resistance to green peach aphids. Plant were less attractive to aphid in the subjective dawn, compared to those in the subjective dusk. Disrupting the circadian clock significantly affects plant resistance to aphid. Gene expression analyses revealed that genes related to regulation and biosynthesis of indolic glucosinolates, a class of plant secondary metabolites promoting defense against aphids, were controlled by clock. Further LC-MS analysis showed altered levels of three major indolic glucosinolates (I3M, 1MO-I3M, and 4MO-I3M) in plants with dysfunctional circadian clock, indicating a novel function of circadian clock in indolic glucosinolate biosynthesis. In summary, our data revealed for the first time a direct role of the circadian clock in plant defense against aphids.

Together these results contribute novel understanding to pathways related to plant defense against aphids via identifying key regulators and elucidating the

mechanisms of plant resistance. Additional, the findings pave the way for future development of new tools for pest management.

REFERENCES

Abuqamar S, Chai MF, Luo H, Song F, Mengiste T (2008) Tomato protein kinase 1b mediates signaling of plant responses to necrotrophic fungi and insect herbivory. Plant Cell **20:** 1964-1983

Aksoy E, Jeong IS, Koiwa H (2013) Loss of function of *Arabidopsis* C-terminal domain phosphatase-like1 activates iron deficiency responses at the transcriptional level. Plant Physiol **161:** 330-345

Alabadi D, Oyama T, Yanovsky MJ, Harmon FG, Mas P, Kay SA (2001) Reciprocal regulation between TOC1 and LHY/CCA1 within the *Arabidopsis* circadian clock. Science **293:** 880-883

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol **55:** 373-399

Awmack CS, Leather SR (2002) Host plant quality and fecundity in herbivorous insects. Annu Rev Entomol **47:** 817-844

Bak S, Tax FE, Feldmann KA, Galbraith DW, Feyereisen R (2001) CYP83B1, a cytochrome P450 at the metabolic branch point in auxin and indole glucosinolate biosynthesis in *Arabidopsis*. Plant Cell **13:** 101-111

Barah P, Winge P, Kusnierczyk A, Tran DH, Bones AM (2013) Molecular signatures in *Arabidopsis thaliana* in response to insect attack and bacterial infection. Plos One **8:** e58987

Barak S, Tobin EM, Andronis C, Sugano S, Green RM (2000) All in good time: the *Arabidopsis* circadian clock. Trends Plant Sci **5:** 517-522

Baudry A, Ito S, Song YH, Strait AA, Kiba T, Lu S, Henriques R, Pruneda-Paz JL, Chua NH, Tobin EM, Kay SA, Imaizumi T (2010) F-box proteins FKF1 and LKP2 act in concert with ZEITLUPE to control *Arabidopsis* clock progression. Plant Cell **22:** 606-622

Bhardwaj V, Meier S, Petersen LN, Ingle RA, Roden LC (2011) Defence responses of *Arabidopsis thaliana* to infection by *Pseudomonas syringae* are regulated by the circadian clock. PLoS One **6:** e26968

Bi JL, Felton GW (1995) Foliar oxidative stress and insect herbivory - primary compounds, secondary metabolites, and reactive oxygen species as components of induced resistance. J Chem Ecol **21:** 1511-1530

Binder BM, Walker JM, Gagne JM, Emborg TJ, Hemmann G, Bleecker AB, Vierstra RD (2007) The *Arabidopsis* EIN3 binding F-box proteins EBF1 and EBF2 have distinct but overlapping roles in ethylene signaling. Plant Cell **19:** 509-523

Blasing OE, Gibon Y, Gunther M, Hohne M, Morcuende R, Osuna D, Thimm O, Usadel B, Scheible WR, Stitt M (2005) Sugars and circadian regulation make major contributions to the global regulation of diurnal gene expression in *Arabidopsis*. Plant Cell **17:** 3257-3281

Boller T, Felix G (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol **60:** 379-406

Bones AM, Rossiter JT (2006) The enzymic and chemically induced decomposition of glucosinolates. Phytochemistry **67:** 1053-1067

Bos JIB, Prince D, Pitino M, Maffei ME, Win J, Hogenhout SA (2010) A functional genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (Green Peach Aphid). Plos Genetics **6:** e1001216

Bouchez O, Huard C, Lorrain S, Roby D, Balague C (2007) Ethylene is one of the key elements for cell death and Defense response control in the *Arabidopsis* lesion mimic mutant *vad1*. Plant Physiol **145:** 465-477

Brader G, Tas E, Palva ET (2001) Jasmonate-dependent induction of indole glucosinolates in *Arabidopsis* by culture filtrates of the nonspecific pathogen *Erwinia carotovora*. Plant Physiol **126:** 849-860

Brattsten LB (1988) Enzymic adaptations in leaf-feeding insects to host-plant allelochemicals. J Chem Ecol **14:** 1919-1939

Broekaert WF, Delaure SL, De Bolle MF, Cammue BP (2006) The role of ethylene in host-pathogen interactions. Annu Rev Phytopathol **44:** 393-416

Bujdoso N, Davis SJ (2013) Mathematical modeling of an oscillating gene circuit to unravel the circadian clock network of *Arabidopsis thaliana*. Front Plant Sci **4:** 3

Celenza JL, Quiel JA, Smolen GA, Merrikh H, Silvestro AR, Normanly J, Bender J (2005) The *Arabidopsis* ATR1 Myb transcription factor controls indolic glucosinolate homeostasis. Plant Physiol **137:** 253-262

Chen HM, Xue L, Chintamanani S, Germain H, Lin HQ, Cui HT, Cai R, Zuo JR, Tang XY, Li X, Guo HW, Zhou JM (2009) ETHYLENE INSENSITIVE3 and ETHYLENE INSENSITIVE3-LIKE1 repress SALICYLIC ACID INDUCTION DEFICIENT2 expression to negatively regulate plant innate immunity in *Arabidopsis*. Plant Cell **21:** 2527-2540

Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nurnberger T, Jones JDG, Felix G, Boller T (2007) A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. Nature **448:** 497-500

Clay NK, Adio AM, Denoux C, Jander G, Ausubel FM (2009) Glucosinolate metabolites required for an *Arabidopsis* innate immune response. Science **323:** 95-101

Cohn JR, Martin GB (2005) *Pseudomonas syringae pv. tomato* type III effectors AvrPto and AvrPtoB promote ethylene-dependent cell death in tomato. Plant Journal **44:** 139-154

Cooper WR, Goggin FL (2005) Effects of jasmonate-induced defenses in tomato on the potato aphid, *Macrosiphum euphorbiae*. Entomol Exp Appl **115:** 107-115

Couldridge C, Newbury HJ, Ford-Lloyd B, Bale J, Pritchard J (2007) Exploring plant responses to aphid feeding using a full *Arabidopsis* microarray reveals a

small number of genes with significantly altered expression. Bull Entomol Res **97:** 523-532

Covington MF, Maloof JN, Straume M, Kay SA, Harmer SL (2008) Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. Genome Biol **9:** R130

Dadd RH (1967) Improvement of synthetic diet for the aphid *Myzus persicae* using plant juices, nucleic acids, or trace metals. J Insect Physiol **13:** 763-778

De Vos M, Jander G (2009) *Myzus persicae* (green peach aphid) salivary components induce defence responses in *Arabidopsis thaliana*. Plant Cell Environ **32:** 1548-1560

De Vos M, Van Oosten VR, Van Poecke RMP, Van Pelt JA, Pozo MJ, Mueller MJ, Buchala AJ, Metraux JP, Van Loon LC, Dicke M, Pieterse CMJ (2005) Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. Mol Plant Microbe In **18:** 923-937

DeJong RJ, Miller LM, Molina-Cruz A, Gupta L, Kumar S, Barillas-Mury C (2007) Reactive oxygen species detoxification by catalase is a major determinant of fecundity in the mosquito *Anopheles gambiae*. Proc Natl Acad Sci USA **104:** 2121-2126

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E, Ryals J (1994) A central role of salicylic acid in plant disease resistance. Science **266:** 1247-1250

Despres L, David JP, Gallet C (2007) The evolutionary ecology of insect resistance to plant chemicals. Trends Ecol Evol **22:** 298-307

Dodd AN, Salathia N, Hall A, Kevei E, Toth R, Nagy F, Hibberd JM, Millar AJ, Webb AA (2005) Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. Science **309:** 630-633

Doherty CJ, Kay SA (2010) Circadian control of global gene expression patterns. Annu Rev Genet **44:** 419-444

Dong HP, Peng J, Bao Z, Meng X, Bonasera JM, Chen G, Beer SV, Dong H (2004) Downstream divergence of the ethylene signaling pathway for harpin-stimulated *Arabidopsis* growth and insect defense. Plant Physiol **136:** 3628-3638

Dorschner KW, Ryan JD, Johnson RC, Eikenbary RD (1987) Modification of host nitrogen levels by the greenbug (Homoptera, Aphididae) - its role in resistance of winter-wheat to aphids. Environ Entomol **16:** 1007-1011

Ellis C, Karafyllidis I, Turner JG (2002) Constitutive activation of jasmonate signaling in an *Arabidopsis* mutant correlates with enhanced resistance to *Erysiphe cichoracearum*, *Pseudomonas syringae*, and *Myzus persicae*. Mol Plant Microbe Interact **15:** 1025-1030

Espinoza C, Degenkolbe T, Caldana C, Zuther E, Leisse A, Willmitzer L, Hincha DK, Hannah MA (2010) Interaction with diurnal and circadian regulation results in dynamic metabolic and transcriptional changes during cold acclimation in *Arabidopsis*. PLoS One **5:** e14101

Farre EM, Weise SE (2012) The interactions between the circadian clock and primary metabolism. Curr Opin Plant Biol **15:** 293-300

Finlayson SA, Hays DB, Morgan P (2007) *phyB-1* Sorghum maintains responsiveness to simulated shade, irradiance and red light: far-red light. Plant Cell Environ **30:** 952-962

Francis F, Vanhaelen N, Haubruge E (2005) Glutathione S-transferases in the adaptation to plant secondary metabolites in the *Myzus persicae* aphid. Arch Insect Biochem **58:** 166-174

Fujita M, Fujita Y, Noutoshi Y, Takahashi F, Narusaka Y, Yamaguchi-Shinozaki K, Shinozaki K (2006) Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Curr Opin Plant Biol **9:** 436-442

Fukushima A, Kusano M, Nakamichi N, Kobayashi M, Hayashi N, Sakakibara H, Mizuno T, Saito K (2009) Impact of clock-associated *Arabidopsis* pseudo-response regulators in metabolic coordination. Proc Natl Acad Sci USA **106:** 7251-7256

Gendron JM, Pruneda-Paz JL, Doherty CJ, Gross AM, Kang SE, Kay SA (2012) *Arabidopsis* circadian clock protein, TOC1, is a DNA-binding transcription factor. Proc Natl Acad Sci USA **109:** 3167-3172

Georghiou GP, Lagunes-Tejeda A (1991) The occurrence of resistance to pesticides in arthropods. Food and Agriculture Organization of the United Nations, Rome. 318 pp.

Girousse C, Moulia B, Silk W, Bonnemain JL (2005) Aphid infestation causes different changes in carbon and nitrogen allocation in alfalfa stems as well as different inhibitions of longitudinal and radial expansion. Plant Physiol **137:** 1474-1484

Goggin FL (2007) Plant-aphid interactions: molecular and ecological perspectives. Curr Opin Plant Biol **10:** 399-408

Gomez-Gomez L, Boller T (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Mol Cell **5:** 1003-1011

Gomeza SK, Oosterhuisb DM, Hendrixc DL, Johnsond DR, Steinkrause DC (2006) Diurnal pattern of aphid feeding and its effect on cotton leaf physiology. Environ Exp Bot **55:** 77-86

Goodspeed D, Chehab EW, Min-Venditti A, Braam J, Covington MF (2012) *Arabidopsis* synchronizes jasmonate-mediated defense with insect circadian behavior. Proc Natl Acad Sci USA **109:** 4674-4677

Goodspeed D, Liu JD, Chehab EW, Sheng Z, Francisco M, Kliebenstein DJ, Braam J (2013) Postharvest circadian entrainment enhances crop pest resistance and phytochemical cycling. Curr Biol **23:** 1235-1241

Griebel T, Zeier J (2008) Light regulation and daytime dependency of inducible plant defenses in *Arabidopsis*: phytochrome signaling controls systemic acquired resistance rather than local defense. Plant Physiol **147:** 790-801

Griffiths DW, Deighton N, Birch ANE, Patrian B, Baur R, Stadler E (2001) Identification of glucosinolates on the leaf surface of plants from the Cruciferae and other closely related species. Phytochemistry **57:** 693-700

Guo H, Ecker JR (2004) The ethylene signaling pathway: new insights. Curr Opin Plant Biol **7:** 40-49

Guo R, Shen W, Qian H, Zhang M, Liu L, Wang Q (2013) Jasmonic acid and glucose synergistically modulate the accumulation of glucosinolates in *Arabidopsis thaliana*. J Exp Bot **64:** 5707-5719

Gutierrez RA, Stokes TL, Thum K, Xu X, Obertello M, Katari MS, Tanurdzic M, Dean A, Nero DC, McClung CR, Coruzzi GM (2008) Systems approach identifies an organic nitrogen-responsive gene network that is regulated by the master clock control gene *CCA1*. Proc Natl Acad Sci USA **105:** 4939-4944

Hall A, Bastow RM, Davis SJ, Hanano S, McWatters HG, Hibberd V, Doyle MR, Sung S, Halliday KJ, Amasino RM, Millar AJ (2003) The *TIME FOR COFFEE* gene maintains the amplitude and timing of *Arabidopsis* circadian clocks. Plant Cell **15:** 2719-2729

Hanano S, Domagalska MA, Nagy F, Davis SJ (2006) Multiple phytohormones influence distinct parameters of the plant circadian clock. Genes Cells **11:** 1381-1392

Hao PY, Liu CX, Wang YY, Chen RZ, Tang M, Du B, Zhu LL, He G (2008) Herbivore-induced callose deposition on the sieve plates of rice: an important mechanism for host resistance. Plant Physiol **146:** 1810-1820

Harmer SL, Hogenesch JB, Straume M, Chang HS, Han B, Zhu T, Wang X, Kreps JA, Kay SA (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science **290:** 2110-2113

Harmer SL, Kay SA (2005) Positive and negative factors confer phase-specific circadian regulation of transcription in *Arabidopsis*. Plant Cell **17:** 1926-1940

Haydon MJ, Mielczarek O, Robertson FC, Hubbard KE, Webb AA (2013) Photosynthetic entrainment of the *Arabidopsis thaliana* circadian clock. Nature **502:** 689-692

Hayes JD, McLellan LI (1999) Glutathione and glutathione-dependent enzymes represent a Co-ordinately regulated defence against oxidative stress. Free Radical Res **31:** 273-300

Hayes JD, Pulford DJ (1995) The glutathione S-Transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol Biol **30:** 445-600

Hazen SP, Schultz TF, Pruneda-Paz JL, Borevitz JO, Ecker JR, Kay SA (2005) LUX ARRHYTHMO encodes a Myb domain protein essential for circadian rhythms. Proc Natl Acad Sci USA **102:** 10387-10392

Heath MC (2000) Hypersensitive response-related death. Plant Mol Biol **44:** 321-334

Heil M, Baldwin IT (2002) Fitness costs of induced resistance: emerging experimental support for a slippery concept. Trends Plant Sci **7:** 61-67

Helfer A, Nusinow DA, Chow BY, Gehrke AR, Bulyk ML, Kay SA (2011) LUX ARRHYTHMO encodes a nighttime repressor of circadian gene expression in the *Arabidopsis* core clock. Curr Biol **21:** 126-133

Hodge S, Ward JL, Beale MH, Bennett M, Mansfield JW, Powell G (2013) Aphid-induced accumulation of trehalose in *Arabidopsis thaliana* is systemic and dependent upon aphid density. Planta **237:** 1057-1064

Hoeberichts FA, Woltering EJ (2003) Multiple mediators of plant programmed cell death: interplay of conserved cell death mechanisms and plant-specific regulators. Bioessays **25:** 47-57

Hogenhout SA, Ammar el D, Whitfield AE, Redinbaugh MG (2008) Insect vector interactions with persistently transmitted viruses. Annu Rev Phytopathol **46:** 327-359

Hopkins RJ, van Dam NM, van Loon JJ (2009) Role of glucosinolates in insect-plant relationships and multitrophic interactions. Annu Rev Entomol **54:** 57-83

Howe GA, Jander G (2008) Plant immunity to insect herbivores. Annu Rev Plant Biol **59:** 41-66

Huang W, Perez-Garcia P, Pokhilko A, Millar AJ, Antoshechkin I, Riechmann JL, Mas P (2012) Mapping the core of the *Arabidopsis* circadian clock defines the network structure of the oscillator. Science **336:** 75-79

Hunt E, Gattolin S, Newbury HJ, Bale JS, Tseng HM, Barrett DA, Pritchard J (2010) A mutation in amino acid permease AAP6 reduces the amino acid content

of the *Arabidopsis* sieve elements but leaves aphid herbivores unaffected. J Exp Bot **61:** 55-64

Hunt EJ, Pritchard J, Bennett MJ, Zhu X, Barrett DA, Allen T, Bale JS, Newbury HJ (2006) The *Arabidopsis thaliana Myzus persicae* model system demonstrates that a single gene can influence the interaction between a plant and a sap-feeding insect. Mol Ecol **15:** 4203-4213

Hunt MD, Delaney TP, Dietrich RA, Weymann KB, Dangl JL, Ryals JA (1997) Salicylate-independent lesion formation in *Arabidopsis lsd* mutants. Mol Plant Microbe In **10:** 531-536

Jirage D, Tootle TL, Reuber TL, Frost LN, Feys BJ, Parker JE, Ausubel FM, Glazebrook J (1999) *Arabidopsis thaliana PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. Proc Natl Acad Sci USA **96:** 13583-13588

Jones JD, Dangl JL (2006) The plant immune system. Nature **444:** 323-329

Kaloshian I (2004) Gene-for-gene disease resistance: bridging insect pest and pathogen defense. J Chem Ecol **30:** 2419-2438

Kerchev PI, Karpinska B, Morris JA, Hussain A, Verrall SR, Hedley PE, Fenton B, Foyer CH, Hancock RD (2013) VITAMIN C and the ABSCISIC ACID-INSENSITIVE 4 transcription factor are important determinants of aphid resistance in *Arabidopsis*. Antioxid Redox Sign **18:** 2091-2105

Kerwin RE, Jimenez-Gomez JM, Fulop D, Harmer SL, Maloof JN, Kliebenstein DJ (2011) Network quantitative trait loci mapping of circadian clock outputs

identifies metabolic pathway-to-clock linkages in *Arabidopsis*. Plant Cell **23:** 471-485

Kettles GJ, Drurey C, Schoonbeek HJ, Maule AJ, Hogenhout SA (2013) Resistance of *Arabidopsis thaliana* to the green peach aphid, *Myzus persicae*, involves camalexin and is regulated by microRNAs. New Phytol **198:** 1178-1190

Kim JH, Jander G (2007) *Myzus persicae* (green peach aphid) feeding on *Arabidopsis* induces the formation of a deterrent indole glucosinolate. Plant Journal **49:** 1008-1019

Kim JH, Lee BW, Schroeder FC, Jander G (2008) Identification of indole glucosinolate breakdown products with antifeedant effects on *Myzus persicae* (green peach aphid). Plant Journal **54:** 1015-1026

Kim WY, Geng R, Somers DE (2003) Circadian phase-specific degradation of the F-box protein ZTL is mediated by the proteasome. Proc Natl Acad Sci USA **100:** 4933-4938

Klingler J, Powell G, Thompson GA, Isaacs R (1998) Phloem specific aphid resistance in Cucumis melo line AR5: effects on feeding behaviour and performance of *Aphis gossypii*. Entomol Exp Appl **86:** 79–88

Klingler JP, Nair RM, Edwards OR, Singh KB (2009) A single gene, *AIN*, in *Medicago truncatula* mediates a hypersensitive response to both bluegreen aphid and pea aphid, but confers resistance only to bluegreen aphid. J Exp Bot **60:** 4115-4127

Koornneef A, Pieterse CMJ (2008) Cross talk in defense signaling. Plant Physiol **146:** 839-844

Kusnierczyk A, Winge P, Jorstad TS, Troczynska J, Rossiter JT, Bones AM (2008) Towards global understanding of plant defence against aphids - timing and dynamics of early *Arabidopsis* defence responses to cabbage aphid (*Brevicoryne brassicae*) attack. Plant Cell Environ **31:** 1097-1115

Kusnierczyk A, Winge P, Midelfart H, Armbruster WS, Rossiter JT, Bones AM (2007) Transcriptional responses of *Arabidopsis thaliana* ecotypes with different glucosinolate profiles after attack by polyphagous *Myzus persicale* and oligophagous *Brevicoryne brassicae*. J Exp Bot **58:** 2537-2552

Lai AG, Doherty CJ, Mueller-Roeber B, Kay SA, Schippers JH, Dijkwel PP (2012) CIRCADIAN CLOCK-ASSOCIATED 1 regulates ROS homeostasis and oxidative stress responses. Proc Natl Acad Sci USA **109:** 17129-17134

Laluk K, Luo H, Chai M, Dhawan R, Lai Z, Mengiste T (2011) Biochemical and genetic requirements for function of the immune response regulator *BOTRYTIS*-INDUCED KINASE1 in plant growth, ethylene signaling, and PAMP-triggered immunity in *Arabidopsis*. Plant Cell **23:** 2831-2849

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Phys **48:** 251-275

Lei J, Finlayson SA, Salzman RA, Shan L, Zhu-Salzman K (2014) *BOTRYTIS*-INDUCED KINASE1 Modulates *Arabidopsis* Resistance to Green Peach Aphids via PHYTOALEXIN DEFICIENT4. Plant Physiol **165:** 1657-1670

Li X, Schuler MA, Berenbaum MR (2007) Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annu Rev Entomol **52:** 231-253

Lin W, Lu D, Gao X, Jiang S, Ma X, Wang Z, Mengiste T, He P, Shan L (2013) Inverse modulation of plant immune and brassinosteroid signaling pathways by the receptor-like cytoplasmic kinase BIK1. Proc Natl Acad Sci USA **110:** 12114-12119

Liu X, Williams CE, Nemacheck JA, Wang H, Subramanyam S, Zheng C, Chen MS (2010) Reactive oxygen species are involved in plant defense against a gall midge. Plant Physiol **152:** 985-999

Liu Z, Wu Y, Yang F, Zhang Y, Chen S, Xie Q, Tian X, Zhou JM (2013) BIK1 interacts with PEPRs to mediate ethylene-induced immunity. Proc Natl Acad Sci USA **110:** 6205-6210

Lorrain S, Vailleau F, Balague C, Roby D (2003) Lesion mimic mutants: keys for deciphering cell death and defense pathways in plants? Trends Plant Sci **8:** 263-271

Louis J, Gobbato E, Mondal HA, Feys BJ, Parker JE, Shah J (2012) Discrimination of *Arabidopsis* PAD4 activities in defense against green peach aphid and pathogens. Plant Physiol **158:** 1860-1872

Louis J, Shah J (2013) *Arabidopsis thaliana-Myzus persicae* interaction: shaping the understanding of plant defense against phloem-feeding aphids. Front Plant Sci **4:** 1-18

Lu DP, Wu SJ, Gao XQ, Zhang YL, Shan LB, He P (2010) A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. Proc Natl Acad Sci USA **107:** 496-501

Luna E, Pastor V, Robert J, Flors V, Mauch-Mani B, Ton J (2011) Callose deposition: a multifaceted plant defense response. Mol Plant Microbe In **24:** 183-193

Mantelin S, Bhattarai KK, Kaloshian I (2009) Ethylene contributes to potato aphid susceptibility in a compatible tomato host. New Phytol **183:** 444-456

Mas P, Kim WY, Somers DE, Kay SA (2003) Targeted degradation of TOC1 by ZTL modulates circadian function in *Arabidopsis thaliana*. Nature **426:** 567-570

McClung CR (2006) Plant circadian rhythms. Plant Cell **18:** 792-803

McClung CR, Gutierrez RA (2010) Network news: prime time for systems biology of the plant circadian clock. Curr Opin Genet Dev **20:** 588-598

Mewis I, Appel HM, Hom A, Raina R, Schultz JC (2005) Major signaling pathways modulate *Arabidopsis* glucosinolate accumulation and response to both phloem-feeding and chewing insects. Plant Physiol **138:** 1149-1162

Mewis I, Appel HM, Hom A, Raina R, Schultz JC (2005) Major signaling pathways modulate *Arabidopsis* glucosinolate accumulation and response to both phloem-feeding and chewing insects. Plant Physiol **138:** 1149-1162

Mewis I, Schreiner M, Nguyen CN, Krumbein A, Ulrichs C, Lohse M, Zrenner R (2012) UV-B irradiation changes specifically the secondary metabolite profile in

broccoli sprouts: induced Signaling overlaps with defense response to biotic stressors. Plant Cell Physiol **53:** 1546-1560

Mewis I, Tokuhisa JG, Schultz JC, Appel HM, Ulrichs C, Gershenzon J (2006) Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. Phytochemistry **67:** 2450-2462

Miller G, Schlauch K, Tam R, Cortes D, Torres MA, Shulaev V, Dangl JL, Mittler R (2009) The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. Science Signaling **2:** 1-10

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci **9:** 490-498

Mizoguchi T, Wheatley K, Hanzawa Y, Wright L, Mizoguchi M, Song HR, Carre IA, Coupland G (2002) LHY and CCA1 are partially redundant genes required to maintain circadian rhythms in *Arabidopsis*. Developmental Cell **2:** 629-641

Mohase L, van der Westhuizen AJ (2002) Salicylic acid is involved in resistance responses in the Russian wheat aphid-wheat interaction. J Plant Physiol **159:** 585-590

Monaghan J, Zipfel C (2012) Plant pattern recognition receptor complexes at the plasma membrane. Curr Opin Plant Biol **15:** 349-357

Moran PJ, Cheng Y, Cassell JL, Thompson GA (2002) Gene expression profiling of *Arabidopsis thaliana* in compatible plant-aphid interactions. Arch Insect Biochem Physiol **51:** 182-203

Moran PJ, Thompson GA (2001) Molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways. Plant Physiol **125:** 1074-1085

Nandi A, Kachroo P, Fukushige H, Hildebrand DF, Klessig DF, Shah J (2003) Ethylene and jasmonic acid signaling affect the NPR1-independent expression of defense genes without impacting resistance to *Pseudomonas syringae* and *Peronospora parasitica* in the *Arabidopsis ssi1* mutant. Mol Plant Microbe In **16:** 588-599

Nisbet AJ, Woodford JAT, Strang RHC (1994) Quantifying aphid feeding on non-radioactive food sources. Entomol Exp Appl **72:** 85-89

Nombela G, Williamson VM, Muniz M (2003) The root-knot nematode resistance gene *Mi-1.2* of tomato is responsible for resistance against the whitefly *Bemisia tabaci*. Mol Plant Microbe In **16:** 645-649

Novakova M, Motyka V, Dobrev PI, Malbeck J, Gaudinova A, Vankova R (2005) Diurnal variation of cytokinin, auxin and abscisic acid levels in tobacco leaves. J Exp Bot **56:** 2877-2883

O'Connor TR, Dyreson C, Wyrick JJ (2005) Athena: a resource for rapid visualization and systematic analysis of *Arabidopsis* promoter sequences. Bioinformatics **21:** 4411-4413

Pegadaraju V, Knepper C, Reese J, Shah J (2005) Premature leaf senescence modulated by the *Arabidopsis PHYTOALEXIN DEFICIENT4* gene is associated with defense against the phloem-feeding green peach aphid. Plant Physiol **139:** 1927-1934

Pegadaraju V, Louis J, Singh V, Reese JC, Bautor J, Feys BJ, Cook G, Parker JE, Shah J (2007) Phloem-based resistance to green peach aphid is controlled by *Arabidopsis PHYTOALEXIN DEFICIENT4* without its signaling partner *ENHANCED DISEASE SUSCEPTIBILITY1*. Plant Journal **52:** 332-341

Prince DC, Drurey C, Zipfel C, Hogenhout SA (2014) The leucine-rich repeat receptor-like kinase BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1 and the cytochrome P450 PHYTOALEXIN DEFICIENT3 contribute to innate immunity to aphids in *Arabidopsis*. Plant Physiol **164:** 2207-2219

Pruneda-Paz JL, Kay SA (2010) An expanding universe of circadian networks in higher plants. Trends Plant Sci **15:** 259-265

Rask L, Andreasson E, Ekbom B, Eriksson S, Pontoppidan B, Meijer J (2000) Myrosinase: gene family evolution and herbivore defense in Brassicaceae. Plant Mol Biol **42:** 93-113

Rasmann S, De Vos M, Casteel CL, Tian D, Halitschke R, Sun JY, Agrawal AA, Felton GW, Jander G (2012) Herbivory in the previous generation primes plants for enhanced insect resistance. Plant Physiol **158:** 854-863

Rentel MC, Lecourieux D, Ouaked F, Usher SL, Petersen L, Okamoto H, Knight H, Peck SC, Grierson CS, Hirt H, Knight MR (2004) OXI1 kinase is necessary for oxidative burst-mediated signalling in *Arabidopsis*. Nature **427:** 858-861

Rossi M, Goggin FL, Milligan SB, Kaloshian I, Ullman DE, Williamson VM (1998) The nematode resistance gene *Mi* of tomato confers resistance against the potato aphid. Proc Natl Acad Sci USA **95:** 9750-9754

Schaffer R, Ramsay N, Samach A, Corden S, Putterill J, Carre IA, Coupland G (1998) The late elongated hypocotyl mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. Cell **93:** 1219-1229

Seo PJ, Mas P (2014) Multiple layers of posttranslational regulation refine circadian clock activity in *Arabidopsis*. Plant Cell **26:** 79-87

Singh V, Louis J, Ayre BG, Reese JC, Pegadaraju V, Shah J (2011) TREHALOSE PHOSPHATE SYNTHASE11-dependent trehalose metabolism promotes *Arabidopsis thaliana* defense against the phloem-feeding insect *Myzus persicae*. Plant Journal **68:** 938-938

Sonderby IE, Geu-Flores F, Halkier BA (2010) Biosynthesis of glucosinolates - gene discovery and beyond. Trends Plant Sci **15:** 283-290

Staiger D, Green R (2011) RNA-based regulation in the plant circadian clock. Trends Plant Sci **16:** 517-523

Stewart SA, Hodge S, Ismail N, Mansfield JW, Feys BJ, Prosperi JM, Huguet T, Ben C, Gentzbittel L, Powell G (2009) The *RAP1* gene confers effective, race-specific resistance to the pea aphid in *Medicago truncatula* independent of the hypersensitive reaction. Mol Plant Microbe In **22:** 1645-1655

Stumpf N, Nauen R (2002) Biochemical markers linked to abamectin resistance in *Tetranychus urticae* (Acari : Tetranychidae). Pestic Biochem Phys **72:** 111-121

Takada H (1986) Genotypic composition and insecticide resistance of Japanese populations of *Myzus-Persicae* (Sulzer) (Hom, Aphididae). J Appl Entomol **102:** 19-38

Thain SC, Vandenbussche F, Laarhoven LJ, Dowson-Day MJ, Wang ZY, Tobin EM, Harren FJ, Millar AJ, Van Der Straeten D (2004) Circadian rhythms of ethylene emission in *Arabidopsis*. Plant Physiol **136:** 3751-3761

Thompson GA, Goggin FL (2006) Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects. J Exp Bot **57:** 755-766

Tjallingii WF (2006) Salivary secretions by aphids interacting with proteins of phloem wound responses. J Exp Bot **57:** 739-745

Torres MA, Dangl JL, Jones JDG (2002) *Arabidopsis gp91*(phox) homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc Natl Acad Sci USA **99:** 517-522

Torres MA, Jones JD, Dangl JL (2006) Reactive oxygen species signaling in response to pathogens. Plant Physiol **141:** 373-378

Van Breusegem F, Dat JF (2006) Reactive oxygen species in plant cell death. P Plant Physiol **141:** 384-390

Van Emden HF, Harrington R (2007) Aphids as crop pests. CABI, Wallingford, UK ; Cambridge, MA. 717pp

Veronese P, Nakagami H, Bluhm B, AbuQamar S, Chen X, Salmeron J, Dietrich RA, Hirt H, Mengiste T (2006) The membrane-anchored *BOTRYTIS-INDUCED KINASE1* plays distinct roles in *Arabidopsis* resistance to necrotrophic and biotrophic pathogens. Plant Cell **18:** 257-273

Vos P, Simons G, Jesse T, Wijbrandi J, Heinen L, Hogers R, Frijters A, Groenendijk J, Diergaarde P, Reijans M, Fierens-Onstenk J, de Both M, Peleman J, Liharska T, Hontelez J, Zabeau M (1998) The tomato *Mi-1* gene confers resistance to both root-knot nematodes and potato aphids. Nat Biotechnol **16:** 1365-1369

Walling LL (2008) Avoiding effective defenses: strategies employed by phloem-feeding insects. Plant Physiol **146:** 859-866

Wang W, Barnaby JY, Tada Y, Li H, Tor M, Caldelari D, Lee DU, Fu XD, Dong X (2011) Timing of plant immune responses by a central circadian regulator. Nature **470:** 110-114

Wang ZY, Tobin EM (1998) Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. Cell **93:** 1207-1217

Weaver LM, Gan S, Quirino B, Amasino RM (1998) A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatment. Plant Mol Biol **37:** 455-469

Weydert CJ, Cullen JJ (2010) Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. Nature Protocols **5:** 51-66

Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature **414:** 562-565

Will T, Tjallingii WF, Thonnessen A, van Bel AJE (2007) Molecular sabotage of plant defense by aphid saliva. Proc Natl Acad Sci USA **104:** 10536-10541

Will T, van Bel AJE (2006) Physical and chemical interactions between aphids and plants. J Exp Bot **57:** 729-737

Williams B, Kabbage M, Kim HJ, Britt R, Dickman MB (2011) Tipping the balance: *Sclerotinia sclerotiorum* secreted oxalic acid suppresses host defenses by manipulating the host redox environment. PLoS Pathog **7:** e1002107

Wilson ACC, Sternberg LDL, Hurley KB (2011) Aphids alter host-plant nitrogen isotope fractionation. Proc Natl Acad Sci USA **108:** 10220-10224

Winde I, Wittstock U (2011) Insect herbivore counteradaptations to the plant glucosinolate-myrosinase system. Phytochemistry **72:** 1566-1575

Yu IC, Parker J, Bent AF (1998) Gene-for-gene disease resistance without the hypersensitive response in *Arabidopsis dnd1* mutant. Proc Natl Acad Sci USA **95:** 7819-7824

Zhang C, Xie Q, Anderson RG, Ng G, Seitz NC, Peterson T, McClung CR, McDowell JM, Kong D, Kwak JM, Lu H (2013) Crosstalk between the circadian clock and innate immunity in *Arabidopsis*. PLoS Pathog **9:** e1003370

Zhang J, Li W, Xiang TT, Liu ZX, Laluk K, Ding XJ, Zou Y, Gao MH, Zhang XJ, Chen S, Mengiste T, Zhang YL, Zhou JM (2010) Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a *Pseudomonas syringae* effector. Cell Host & Microbe **7:** 290-301

Zhou GX, Qi JF, Ren N, Cheng JA, Erb M, Mao BZ, Lou YG (2009) Silencing *OsHI-LOX* makes rice more susceptible to chewing herbivores, but enhances resistance to a phloem feeder. Plant Journal **60:** 638-648

Zhu-Salzman K, Koiwa H, Salzman RA, Shade RE, Ahn JE (2003) Cowpea bruchid *Callosobruchus maculatus* uses a three-component strategy to overcome a plant defensive cysteine protease inhibitor. Insect Mol Biol **12:** 135-145

Zhu-Salzman K, Salzman RA, Ahn JE, Koiwa H (2004) Transcriptional regulation of sorghum defense determinants against a phloem-feeding aphid. Plant Physiol **134:** 420-431

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JDG, Boller T, Felix G (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. Cell **125:** 749-760